ACTIVITY AND MECHANISM OF *ASCOPHYLLUM NODOSUM* EXTRACT INDUCED SALINITY TOLERANCE IN TOMATO

by

Abhinandan Kumar

Submitted in partial fulfilment of the requirements
for the degree of Master of Science

at

Dalhousie University
Halifax, Nova Scotia
June 2014

I dedicate this thesis to my family who has always been the nearest reserves for motivation whenever I was down. Their unconditional love has always motivated me to set higher goals. I also dedicate this thesis to my friend, Palaniappan Ramanathan, who was nearest to me, all through my stay, like a brother.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Salinity affects crop production worldwide. *Ascophyllum nodosum*, a brown marine alga, has been used for decades as a bio-stimulant to promote plant growth and impart tolerance to biotic and abiotic stresses. However, the mechanism(s) of the bio-stimulatory activity of *A. nodosum* extract (ANE) is not well understood.

In vitro experiments were conducted to study the effect of the ethyl acetate fraction of ANE on two week old tomato plants (cv Scotia) grown under salinity stress (100 mM NaCl). The ethyl acetate fraction significantly improved seedling growth and development under salinity stress, i.e. the leaf area and root length of the treated plants improved while the *in-planta* sodium ion concentration decreased. Stimulated seedlings had higher catalase enzyme activity and recorded higher chlorophyll (chl*a*, chl*b* and carotenoids) content than non-supplemented stressed plants. Moreover, when tested on four week old tomato plants in a greenhouse, ANE treated plants showed higher concentrations of potassium ions compared to controls. The results shed light on the complex nature of ANE.

LIST OF ABBREVIATIONS USED

ANE	*Ascophyllum nodosum* Extract
EtOAc-ANE	Ethyl acetate fraction of *Ascophyllum nodosum* Extract
H_2O	Water
CO_2	Carbon dioxide
FAO	Food and Agriculture Organization
NaCl	Sodium Chloride
Cl^-	Chloride ion
$SO4^{3-}$	Sulphate ion
NO_3^-	Nitrate ion
HCO_3^-	Bicarbonate ion
Na^+	Sodium ion
Ca^{2+}	Calcium ion
Mg^{2+}	Magnesium ion
K^+	Potassium ion
ROS	Reactive oxygen species
DNA	Deoxyribonucleic acid
$^1O_2$	Singlet oxygen
$^-O_2^{\cdot}$	Superoxide radical
H_2O_2	Hydrogen peroxide
$OH^{\cdot}$	Hydroxyl Radical
PCD	Programmed cell death
$CO_3^{\cdot -}$	Carbonate Radical

$CO_2^{\cdot -}$	Carbon dioxide anion radical
RO	Mineral oxide
RO_2	Mineral dioxide
HOCl	Hypochlorite
O_3	Ozone molecule
$ONOO^-$	Peroxynitrite ion
O_2NOO^-	Peroxynitric ion
$RO_2^{\cdot}$	Mineral dioxide radical
$HOOCO_2^-$	Peroxymonocarbonate ion
ONOOH	Peroxynitrous acid
RLK	Receptor-like kinases
GPCR	G-protein coupled receptors in plant
InsP	Inositol phosphate
ABA	Abscisic acid
HKT	High-affinity Potassium Transporters
KIRC	K^+ inward-rectifying channel
NSCC	Non-specific cation channels
KORC	K^+ outward-rectifying channel
V-ATPase	Vacuolar type H^+ ATPase
V-PPase	Vacuolar type pyrophosphatase
PIPB	Phosphatidylinositol bisphosphate
SOS	Salt overly sensitive
CBL	Calcineurin B-like protein

NHX	Na^+/H^+ antiporter
PDH45	Pea DNA helicase 45
RD29A	Desiccation-responsive protein 29A
P5CS	△1-pyrroline-5-carboxylate synthetase
EC	Electrical conductivity
GC	Gas Chromatography
HPLC	High performance liquid chromatography
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
IAA	Indole-3-acetic acid
MeOH	methanol
ANOVA	Analysis of Variance
SAS	Statistical Analysis System
HSD	Honestly significant difference
KH_2PO_4	Monopotassium phosphate
K_2HPO_4	Dipotassium phosphate
PVP	Polyvinylpyrrolidone
EDTA	Ethylenediaminetetraacetic acid
BSA	Bovine serum albumin
OD	Optical density
DMSO	Dimethyl sulfoxide
RNA	Ribonucleic acid
RNase	Ribonuclease

ACKNOWLEDGEMENTS

First, I would like to thank my supervisor, Dr. Balakrishnan Prithiviraj, for his guidance, encouragement and patience over the last few years. Thank you for teaching me some of the skills of being a researcher and the responsibilities of graduate student life. Listening to you in the lab meets was motivating. Thank you for the support, guidance and encouragement throughout my thesis project. I would like to thank my committee members, Dr. Yousef Papadopoulos and Dr. Bernhard Benkel, for their continuous encouragement and constructive feedback during my thesis writing.

I would like to acknowledge the Natural Science and Engineering Research Council of Canada (NSERC) for granting the IPS scholarship for this project. I would like to mention the enormous contribution from Dr. Prithiviraj, Dr. Alan T. Critchley (Acadian Seaplants Limited) and Heather Hughes (Research Services) in procuring this prestigious award.

I would like to extend my gratitude to Dr. Alan T. Critchley, Mr. Will Niely, Mrs. Laurel Shishkov, Mr. Jef Archibald, Dayna, Katy Griegoschewski, Leah and Josie for being such wonderful hosts during my stay in Cornwallis, NS. Thank you, Dr. Wally Shishkov, for sharing your enlightening thoughts, whenever we met.

There are so many wonderful people around me to thank, who have touched my life and have made my stay fun filled. I would like to extend my appreciation to my lab members from Marine Bioproducts research laboratory. Thank you, Dr. Saveetha for always being a friend as well as a vital critique. I would like to thank Dr. Jatinder, Dr. Julia and Dr. Tan for giving me learning experience whenever I talked to you. A huge appreciation for the learning experiences I had with my lab mates, Sridhar, Garima, Pramod, Swati, Gayathri and Mullai, whenever I worked with you. Thank you Nadun for the wonderful experience we had working together.

This note would be incomplete if I do not mention Margie, Anne, Daryl, Paul and Gisele for their unconditional assistance whenever I needed it. My heartfelt

appreciations to Mrs. Marg Rovers and Rosalie Madden, who helped me, make sense out of my English.

I would like to thank Mrs. Kalyani Prithiviraj for the delicious food when I was bedridden for months. I will never forget Palaniappan Ramanathan, without whose dedication to see me in good health, I would never have had the chance to finish this thesis sooner. Major thanks to Parmender Singh, Kaushikan Ganesan and Ashok Somalraju for the loyal company which I had with you, at all stages of my stay.

I bow to the Almighty, to my parents, and to everyone who have given me a lesson to learn in life. Everything which seems to be free, costs time. And thus, I owe to everyone who has agreed to spend a little time with me. I would like to acknowledge their anonymous presence in my life. Thank you all.

CHAPTER 1 Introduction

All living organisms on the earth have specific requirements for optimum growth. Life is an outcome of the homeostasis between the internal and external environments. The environments change frequently and unpredictably, perturbing this delicate balance that might lead to “stress”, a term which is not precise but has general connotations (Osmond et al., 1987).

An environmental condition can be optimum for one organism and stressful for another. The crop plants are frequently exposed to environmental stresses such as variation in temperature, soil water deficit, soil mineral deficiencies, and soil salinity. Other stresses include interactions with biotic factors, such as pathogens, insects, weeds and herbivores. Thus, tolerance to various environmental stresses gives a measure of a plant’s primary assimilation processes (CO_2 and mineral uptake), growth (biomass accumulation), survival and yield (Taiz and Zeigler, 2002).

Soil salinity is one of the most commonly faced challenges in present agriculture. Many cultivated crops are sensitive to low salinity levels. Crop plants cultivated on saline soils have reduced growth and yield. The plants are usually challenged to achieve field yield potential under such conditions. Soil salinity is a dynamic characteristic, largely depending on soil inherent mineral and chemical composition. It is affected by fluctuations in water profile and ion influxes of both edaphic and anthropogenic origins. Thus, the ionic profile of soil has been used as an indicator of soil health (Haberern, 1992), and as a measure of suitability for cultivation. The ionic profile guides the adoption of various agricultural soil

management practices followed in cropping systems. Although, crop plants differ in their ability to tolerate salinity stress, it has been difficult to precisely measure salt tolerance in long duration yield studies, which are often associated with unpredictable environmental constraints that could affect the final outcome. Therefore, a range of indices have been developed to assess the level of plant tolerance to such stresses. Some of the methods of determining a plant's ability to tolerate salinity are to measure germination percentage, leaf area changes, root characteristics (length and area), and biomass accumulation under saline conditions (Munns, 2002).

Plants, being sessile, have developed complex mechanisms to deal with various environmental stresses. Research in plant mineral nutrition began more than 150 years ago (Kochian and Lucas, 2014), and several aspects of plant nutrient acquisition, its interaction with other nutrients and their assimilation inside plants are still under investigation. Challenges on improving plant salt tolerance have led to the development of strategies which include engineering (improve drainage, irrigation), genetic improvement (genetic engineering and breeding) and the application of soil amendments (chemical or organic).

For centuries, whole seaweed, or processed or purified concentrates of seaweeds, have been used in agriculture to improve stress tolerance (Crouch et al., 1990) in plants and animals. *Ascophyllum nodosum* and many other types of seaweed, such as *Laminaria, Fucus* and *Ecklonia,* are commonly used. Their growth promoting effects are contributed by naturally occurring bio-stimulatory components, which include essential micronutrients, traces of vitamins, and

complex organic molecules. These molecules have similar functional effects as hormones found in terrestrial plants (Craigie, 2011; Stirk et al., 2003).

The present study builds upon the knowledge and long history of the use of seaweeds, especially *Ascophyllum nodosum,* as organic amendments under various environmental stresses. The project investigated the potential use of *Ascophyllum nodosum* extract to impart salinity tolerance to plants, using tomato as a model.

CHAPTER 2 Review of Literature

2.1 Soil salinity: a major environmental stress to plants

Soil salinization is a major factor that limits crop production and productivity. Salt adversely affects crop growth, development, and production. Globally more than 800 million ha of land are affected by salinity (Qadir et al., 2007), which is approximately 7% (Shabala and Cuin, 2008) of the total world land area (FAO, 2008). About 45 million ha (20%) out of the 230 million ha of irrigated land area in the world are affected by salinity issues. Similarly, 32 million ha of dry land are affected by salinity (Munns, 2002). Moreover, the salinized areas are increasing at a rate of 10% annually due to environmental factors such as low precipitation, high surface evaporation, weathering of native rocks and anthropogenic causes such as irrigation with saline water and poor agricultural practices (Tanji, 1990; Pessarakli and Szabolcs, 1999). Soil salinity broadly covers a spectrum of losses which include decline in crop production, irrigation management system, costs involved in reclamation of soil and hidden losses due to continuous degradation of soil (soil dispersion, erosion). This loss was estimated to be $12 billion a year in the US (Gnassemi et al., 1995). The expansion of agriculture in arid and semi-arid regions, aided by development of irrigation systems, has caused an increase in the secondary salinization due to improper drainage, overuse of fertilizers and use of poor quality water. It is estimated that more than 50% of the arable land will be salinized by the year 2050 (Ashraf, 2009). Saline soils contain sufficient salts to interfere with the normal growth of most crop species. These soils have an electrical conductivity of > 4 dSm^{-1} (~40 mM NaCl) (USDA-ARS, 2008). The

electrical conductivity depends on the concentrations of different ions present in the soil solution (Bui, 2013), most commonly chlorides, sulfates, nitrates and bicarbonates of sodium (Na), calcium (Ca), magnesium (Mg), and potassium (K). In general, higher salinity corresponds to a higher electrical conductivity. A high spatial variation in electrical conductivity is common in saline soils. Most salts in soil are water soluble and thus, are the main sources of natural (primary) salinization (Schofield et al., 2001). Classifications of such soils are shown in **Table 2.1**. Low moisture content and insufficient leaching of soil causes salt accumulation. Such conditions are common in arid and semiarid areas of the world (Schofield and Kirkby, 2003). Thus, salinization is an *in situ* form of soil degradation which is usually associated with low fertility (Schofield et al., 2001).

Table 2.1: Classification of salt-affected soils (Brady and Weil, 2010)

Classification	Electrical Conductivity (dS/m)	Soil pH	Sodium Adsorption Ratio	Soil Physical Condition
Saline	> 4.0	< 8.5	< 13	Normal
Saline-sodic	> 4.0	< 8.5	> 13	Normal
Sodic	< 4.0	> 8.5	> 13	Poor

2.2 Effect of soil salinity on plant growth

Soil salinity adversely affects plants' physiological processes, resulting in slow growth. Based on tolerance to soil salinity, terrestrial plants have been classified into two groups; halophytes and glycophytes (non-halophytes). Although, only 2% of the terrestrial plant species are halophytes, this class includes a large diversity of plants with members from half of all higher plant families (Glenn et al., 1999). Halophytes have evolved adaptive modifications, such as the presence of salt excreting glands, bladders and succulence. The glycophytes have been further classified as sensitive, moderately sensitive, moderately tolerant and tolerant species. All terrestrial crops fit into a classification in each of these groups **(Figure 2.1)**.

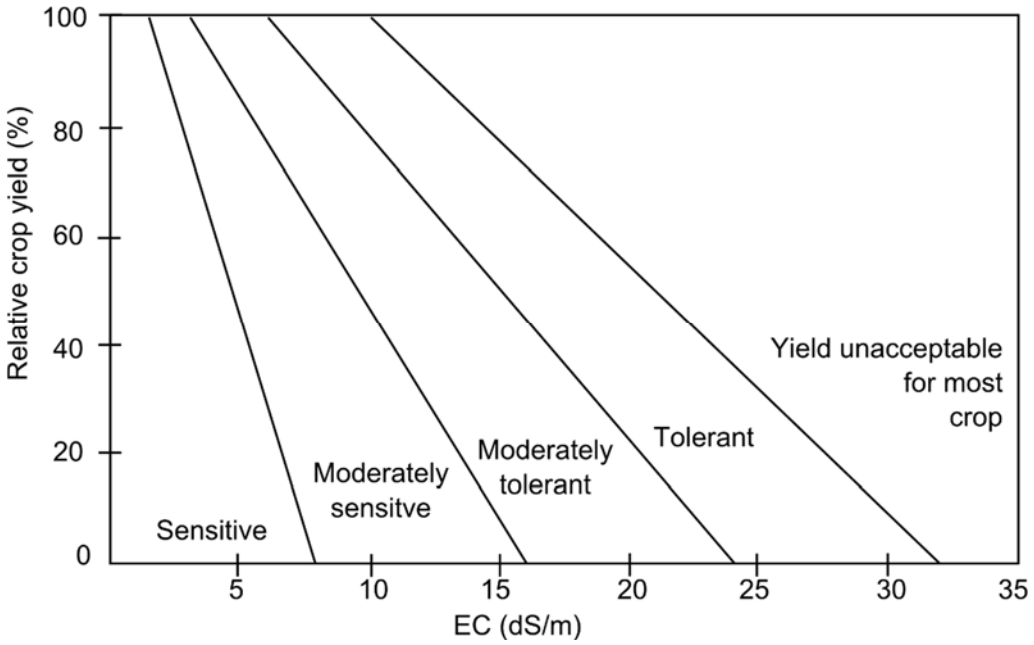


Figure 2.1: Glycophytes- crop tolerance to salinity (Tanji and Kielen, 2002)

Growth suppression, due to suboptimal growth conditions, occurs in all plants. Mohammad et al. (1998) observed a significant reduction in the number of leaves, plant height and biomass in tomato plants, when exposed to increasing concentrations of NaCl (0 - 150 mM). *Halopyrum mucronatum,* a perennial grass, when grown in increasing concentration of NaCl (0 – 360 mM), showed maximum succulence and biomass accumulation at 90 mM NaCl concentration. A further increase in the salinity resulted in death of the plant (Khan et al., 1999). Similarly, *Ceriops roxburghiana*, a mangrove species, accumulated significantly higher biomass with increasing NaCl concentration (upto 400 mM), which then was reduced with further increase in NaCl concentration (Rajesh et al., 1998). Kurban et al. (1999) reported a significant increase in total plant weight at 50 mM (170% more than controls) in a leguminous plant, *Alhagi pseudalhagi,* which was subjected to increasing concentrations (0 – 200 mM) of NaCl. Thus, low salinity

might contribute to stimulatory growth effects, indicating the complex physiochemical pathways involved in plant nutrient acquisition and assimilation, in relation to plant tolerance to salinity. **Figure 2.2,** shows the diverse forms of growth declining effects soil salinity has on plants.

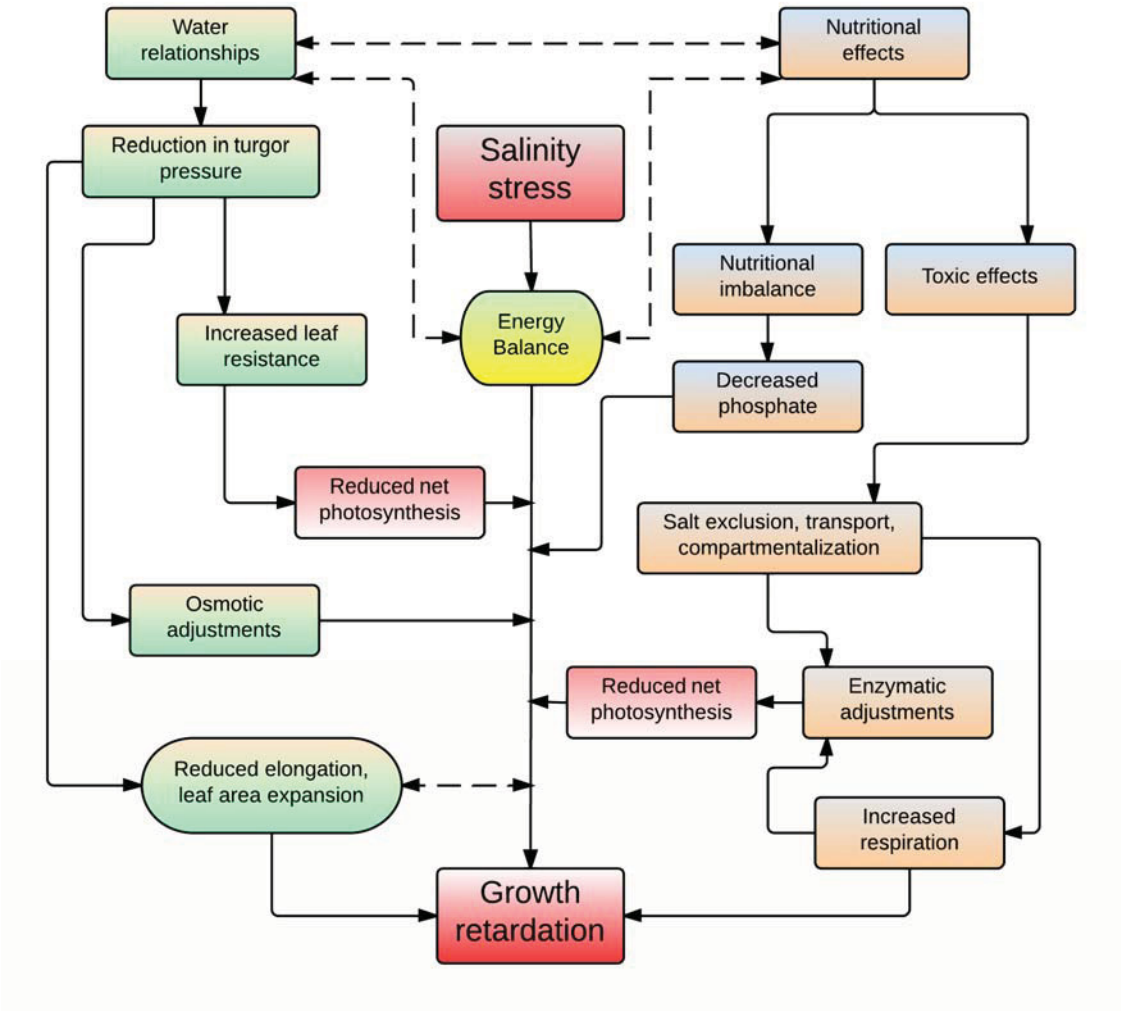


Figure 2.2: Effects of salinity on plant growth (reproduced from Pasternak, 1987)

2.3 Effect of salinity on physiological and biochemical characteristics

2.3.1 Osmotic (water deficit) and ionic dis-equilibrium

The movement of water and nutrients (ions) from soil solution into roots is an electrochemical phenomenon working on the principle of mass flow, osmosis and active acquisition. A large difference in water potential at the soil-plant interface is undesirable. A high concentration of salts in the root zone results in reduced water potential, making it difficult for plants to absorb water. Plants' responses to salinity consist of two physiological phases; the initial phase is the osmotic stress response and this is followed by a specific ion toxicity phase (accumulation of Na^+ and/or Cl^-). The osmotic phase starts immediately after the development of a negative water potential in the rhizosphere.

Water deficit induces abscisic acid (ABA) production, which is primarily involved in regulating stomatal aperture and conductance. Water deficit directly affects net carbon assimilation and photosynthesis (Schroeder et al., 2001; Smith and Stitt, 2007; Kim et al., 2010; Tardieu et al., 2011). Reduced stomatal apertures lower the transpiration rates, which decrease nutrient uptake and increase leaf temperature (Wilkinson and Davies, 2002; Christmann et al., 2007; Kim et al., 2010). A drop in osmotic pressure of the cells is an immediate response to high salt concentration. In barley, short term exposure to high salinity leads to an immediate and significant drop in stomatal conductance, due to osmotic stress and local synthesis of ABA (Fricke et al., 2004; 2006). Low availability of soil water leads to a transient loss of turgor in plants. It reduces cell elongation, which causes a reduction in leaf expansion, as well as other plant parts, ultimately

leading to stunting of the plants (Hernandez et al., 1999). Saline soil, with an EC value of 4 dS/m, generates an osmotic pressure equivalent to 0.2 MPa. A similar osmotic pressure is caused by 40 mM NaCl solution (USDA-ARS. 2008). The growth of most crop plants is hindered beyond this threshold salinity.

Plant have evolved mechanisms to take up essential nutrients such as NO_3^-, K^+, and many other essential micronutrients under unfavorable and disturbed ionic regime commonly encountered in saline soil (Munns and Tester, 2008). NaCl dominates saline soils. High salt concentration destabilizes the membrane polarity of plant cells. It affects selective nutrient absorption and creates ionic imbalances. High Na^+ in soil solution causes intracellular K^+ deficiency due to competition and leads to K^+/Na^+ disequilibrium (Kronzucker and Britto, 2008; Pardo and Rubio, 2011). High concentration of NaCl caused reductions in Ca^{2+} and Mg^{2+} levels in a number of plants (Khan et al., 1999, 2000).

Some woody species, such as Citrus and Vitis (grapevine), show toxic effects of chloride accumulation (White and Broadley, 2001). However, most of the studies have focused on Na^+ acquisition and assimilation, including transport and distribution in plants. Munns and Tester (2008) reported a critical cytosolic threshold level of 100 mM for Na^+ that is detrimental and toxic to most of the enzymes (Flowers and Dalmond, 1992; Tester and Davenport, 2003), although, the range is quite large and based on different methods used for determination of cytosolic concentrations.

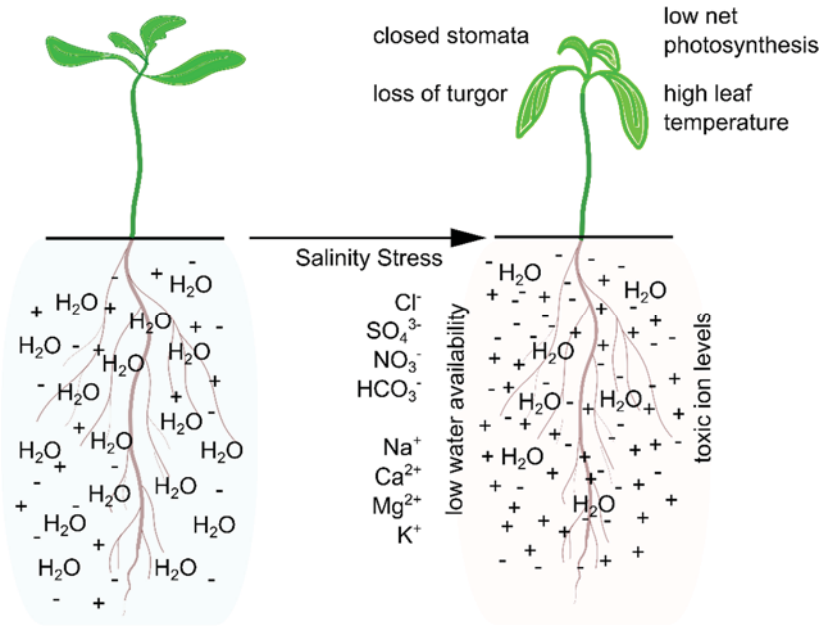


Figure 2.3: Physiological changes in plants due to high salinity

2.3.2 Changes in cell membrane, organelle ultrastructure and leaf anatomy

The plasma membrane of a cell is the first site of cellular interaction with incoming ion fluxes. Ionic movement across membranes is selectively regulated by ion channels and carrier molecules embedded in the membranes. High salinity has been correlated with cell membrane damage leading to leaky cells. Mansour and Stadelmann (1994) reported characteristic changes in membrane permeability of *Hordeum vulgare* cultivars under high salinity conditions (100 mM NaCl). Interestingly, studies on *Hordeum* and *Triticum* showed decreased cytoplasmic viscosity in salt sensitive cultivars which further decreased with an increase in salinity level. In contrast, tolerant lines maintained higher cytoplasmic viscosity even at higher salinity. Thus cytoplasmic characteristics such as permeability and viscosity are important in deciding salt tolerance and vary widely among crop species (Mansour et al., 1993). Cellular organelles show

irreversible morphological changes due to high salinity. Tomato plants exposed to 100 mM exhibited aggregation of chloroplast and loss of thylakoid structures (Khavari-Nejad and Mostofi, 1998). Mitsuya et al. (2000) reported swelling of thylakoid membranes, mitochondria and fragmentation of the tonoplast in sweet potato leaves under 80 mM of NaCl. Similar observations were reported in salt stressed potato plants which resulted in accumulation of large starch grains inside chloroplasts (Bruns and Hecht-Buchholz, 1990). Thus, plants display damage symptoms due to exposure to high salinity. Salinity alters plant physiological processes which lead to characteristics changes in leaf anatomy. The effects of salinity stress on leaf anatomy differ with plant species and specific cell types. Longstreth and Nobel (1979) reported an increase in the thickness of both epidermal and mesophyll cells in *Atriplex* under salinity stress. However, high salinity resulted in a significant decrease in the thickness of mesophyll cells and disorganization of the thylakoids in the leaves of a mangrove, *Bruguiera parviflora* (Parida et al., 2003, 2004). Similarly, spinach leaves were shown to have reduced intercellular space due to salinity. A significant decrease in the stomatal density of tomato plants was recorded when treated with 70 mM of NaCl in a sand culture experiment (Romero-Aranda et al., 2001).

2.3.3 Changes in photosynthesis and production of reactive oxygen species

Photosynthesis is one of the most important physiological processes in the plant. Several external and internal factors affect photosynthetic efficiency. Biomass accumulation in plants is a function of net photosynthesis. Salinity stress impacts growth and developmental processes of plants and directly affects net

photosynthesis. Iyengar and Reddy (1996) explained several factors contributing to decline in photosynthetic rate. A physiological water deficit and a reduction in water potential lowers the photosynthetic efficiency of plants under salinity stress, thus lowering the net carbon assimilation rate. Many reports have indicated reduced photosynthesis under increasing concentrations (0 mM - 100 mM) of NaCl (Romeroaranda et al., 2001; Soussi et al., 1998). A significant decline in the net photosynthesis is an immediate effect of stomatal closure coupled with photorespiration in plants exposed to high salinity stress. This short term response to salinity exposure lasts for 24 – 48 h and completely ceases photosynthesis (Parida et al., 2005).

The long-term effect on net photosynthesis is caused by the accumulation of salts in the growing parts of the plant. High cytosolic concentrations of Na^+ and Cl^- interfere with the optimum activity of several enzymes involved in the carbon assimilation (Munns and Termatt, 1986). Reddy et al. (1992) reported a significant reduction in stomatal conductance and CO_2 assimilation rate in salt stressed *Salicornia brachiata* Roxb. plants which prevented optimal activities of several enzymes in Calvin cycle. Similarly, salt stress aggravated photo-inhibition and delayed recovery of photosynthetic apparatus in wheat cultivars (Mishra et al., 1991). As reviewed by Paul and Foyer (2001), for conservation of the resources and energy, feedback signaling may regulate the rate of photosynthesis due to growth inhibition, and the balance between sources and sinks.

Prolonged exposure to salinity adversely affects the chlorophyll and carotenoid content of leaves. Salinity stress affects the process of chlorophyll synthesis

resulting in chlorophyll being directed to its degradation pathways (Hörtensteiner, 2006). The symptoms of salinity damage include leaf chlorosis (loss of chlorophyll). In most crops, salinity induced chlorosis is first observed in the older leaves (Hernandez et al., 1999). For example, tomato and alfalfa leaves showed a significant reduction in total chlorophyll content, when exposed to salinity levels of 100 mM of NaCl (Khavarinejad and Mostofi, 1998).

Salt stress impairs electron transport processes in organelles, such as chloroplasts and mitochondria, as well as other biochemical pathways. Reactive oxygen species (ROS) are immediately and locally formed charged naïve molecular entities, which are produced within the metabolic pathways of all living organisms. The mitochondria and chloroplast are the most potent sites of ROS production. ROS are the major by-products of the processes involved in quenching and circulation of high energy photons, during low photosynthesis periods and/or unfavorable environmental conditions. ROS are a result of molecular interactions or charge transfers between ionic species. Mittler (2002) and Polle (2001) reported that under normal growth conditions, the ROS concentration in a cell is as low as 240 μMS^{-1} of superoxide, whereas, the steady state level of H_2O_2 in chloroplast is 0.5 µM. Under salinity stress, the ROS level increased three times and the H_2O_2 level increased by 30 times. Thus ROS has been related to many adverse cellular changes. ROS induce autoxidation of fatty acids, proteolysis, peptide fragmentation, amino acid modification and DNA lesions (Smirnoff, 2000; Ahmad et al., 2008; Tuteja et al., 2009). ROS alter membrane fluidity, permeability and susceptibility to damage due to shifting ionic

charges. These changes affect the performance of the plant under various stresses. Environmental stresses, biotic (insects, pathogens) and abiotic (drought, salinity, temperature, light) trigger the production of reactive oxygen species such as singlet oxygen ($^1O_2$), superoxide radical ($^{-}O_2^{\cdot}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$). Photosynthesis, photorespiration and CO_2 assimilation require a basal level of ROS scavenging (Ahmad et al., 2008). Light stress (changing light intensities) produces frequent and rapid changes in the rates of production and consumption of ROS. However, abrupt changes in ROS levels in plants under salinity stress are less distinct (Munns and Tester, 2008), and so plants are able to adjust to slow changes in ROS levels, thereby regaining photosynthesis after a certain period of salt stress imposition. Mansour and Stadelmann (1994) identified genotypic differences in the membrane permeability, including membrane damage or lipid peroxidation due to ROS, of barley cultivars, suggesting possible differences in their inherent tolerance to salts due to genotypic differences (Munns and Tester, 2008).

2.4 Mechanisms of salinity tolerance

Plants are sessile organisms that have evolved sophisticated biochemical and molecular mechanisms to cope with various environmental stresses. Such mechanisms are tightly regulated and coordinated through various signaling pathways. A general approach in response to stimulus or stress conditions is outlined in the **Figure 2.4.**

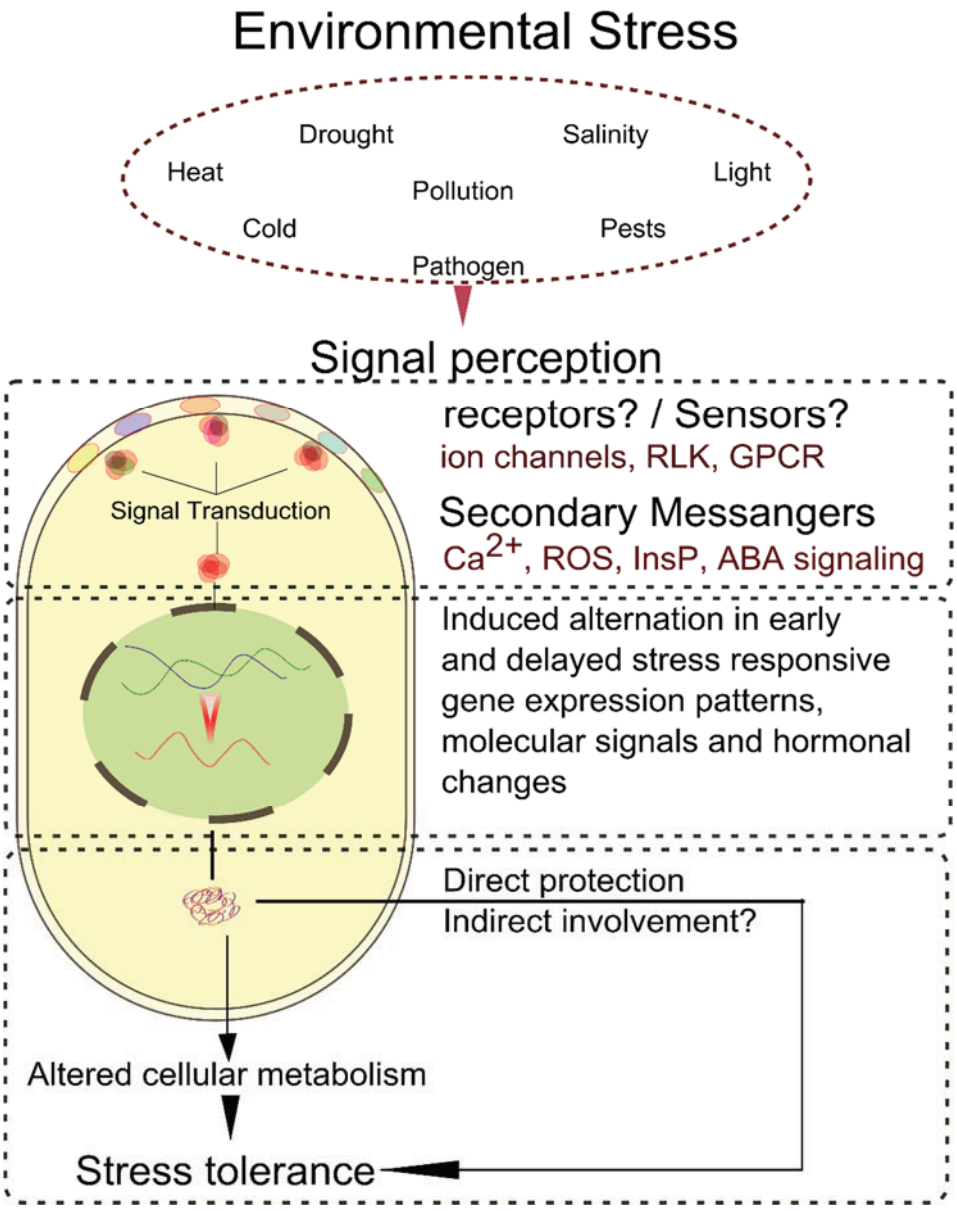


Figure 2.4: A generic response pathway of plants under Stress

The damage due to salinity stress largely depends on the level of salinity, duration of exposure, atmospheric humidity, and soil moisture content. An increase in tolerance to both the osmotic (1st phase) and ionic phases (2nd phase) will enable continued plant growth under salinity stress. Salinity affects plant growth at every stage of plant's life cycle, thus, tolerance at any of

the stages, such as seedling, vegetative, flowering or fruiting, would boost adaptability in salinity stress conditions. Parida et al. (2005) have listed such biochemical mechanisms as: i) the ion regulation phase, which includes accumulation/ exclusion/ compartmentalization or translocation of ions within the plant; and ii) the synthesis phase, which includes synthesis of compatible solutes and alterations in various biochemical pathways leading to salinity tolerance. The outline of the mechanisms involved in salinity tolerance is shown in **Figure 2.5.**

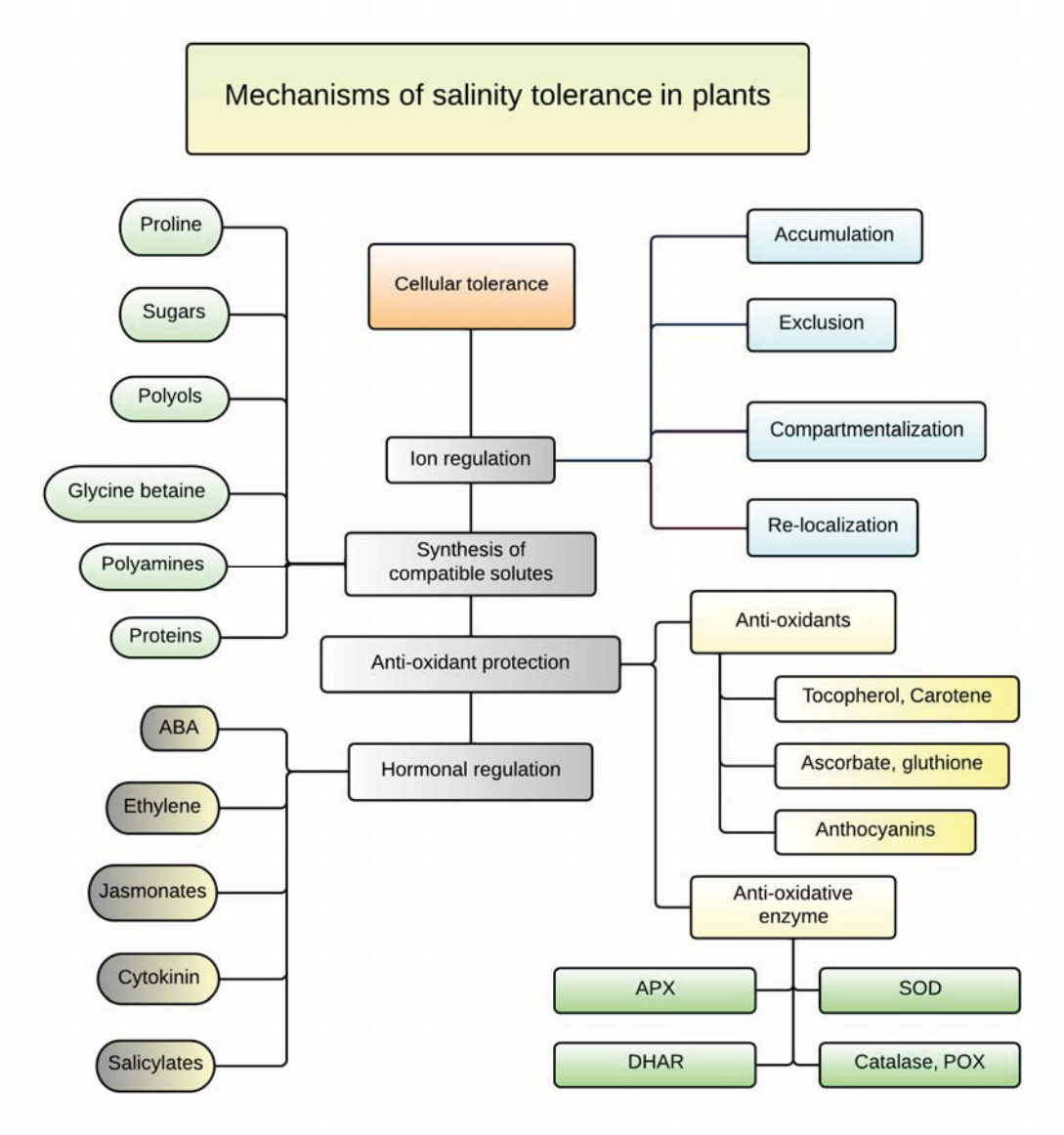


Figure 2.5: Mechanism of salinity tolerance

2.4.1 Ion regulation

High concentrations of salts in the rhizosphere limit plant growth and yield, and may cause death of plants. The entry of ions from the soil to the roots can follow two different pathways; (i) apoplastic, and (ii) symplastic pathways. Symplastic movements of molecules are driven by differential osmotic potentials whereas

apoplastic movements do not require energy for entry. Sodium enters plant cells passively (negative membrane potential) and apoplastically (transpiration pull) (Yeo, 1999), as well as through selective ion transporters, such as High-affinity Potassium Transporters (HKT) and non-selective (cation) channels (Maser et al., 2002; Amtmann and Sanders, 1998).

The mechanism of tolerance during the ionic phase can be further divided into two categories: i) sodium exclusion from leaf blades and, ii) sodium compartmentalization or tissue tolerance to accumulated sodium. Under high salinity, plants sequester the excess ions in subcellular structures such as vacuoles (Iyengar and Reddy, 1996; Reddy et al., 1993). The ion channels regulate the entry of Na^+ or K^+ ions and the competition for an active site increases based on the ionic radii, ionic charges and hydration energies of the molecules. Garciadebleas et al. (2003) demonstrated that, in rice, Ba^{2+} blocked selective entry of Na^+ but not K^+ and that different transporter were involved in Na^+ and K^+ entry into the cells.

The High-affinity Potassium Transporters (HKT) and K^+ inward-rectifying channel (KIRC) have a higher selectivity for K^+ than Na^+ and are involved in K^+ influx. Some nonspecific cation channels (NSCC) and K^+ outward-rectifying channel (KORC) may lead to the accumulation of sodium ions in the cytosol during depolarization. The vacuolar type H^+ ATPase (V-ATPase) and pyrophosphatase (V-PPase) are two electrogenic H^+ pumps which coexist at the plasma membranes of the plants (Dietz et al., 2001). The enzyme, V-ATPase, is an

active, dominant and indispensable H^+ pump, and it is found in the endomembranes of most plants.

Calcium signaling plays an indispensable role in attaining and maintaining stress tolerance. Cytosolic Ca^{2+} increases during salinity stress. The sources for Ca^{2+} can be of apoplastic origin or a hydrolysis product of PIPB (phosphatidylinositol bisphosphate), mediated by phospholipase C. The characterization of salt overly sensitive (SOS) mutants (SOS1, SOS2, SOS3) (Wu et al., 1996) in *Arabidopsis* were a result of the identification of calcium binding proteins such as CBL (calcineurin B-like protein/ SOS3). A loss in function of CBL turns the plants hypersensitive to salts (Zhu, 2002). SOS pathways regulate ion homeostasis. The presence of salt activates SOS pathways which remove excess Na^+ from the cell (Zhang et al., 2004; Zhu, 2002) and also work with the NHX pumps to compartmentalize Na^+ into the vacuoles before Na^+ reaches to toxic level for normal enzymatic activities (Blumwald et al., 2000). The vacuolar Na^+/H^+ pumps sequester excess Na^+ into the vacuoles. Compartmentalization of Na^+ in the vacuoles from the cytoplasm is carried out the by salt-inducible Na^+/H^+ antiporter system (Apse et al., 1999). Staal et al. 1991 reported relatively higher Na^+/H^+ antiporter activity in salt tolerant species than sensitive species of *Plantago*. *nhx-1* overexpression increased salinity tolerance in *Arabidopsis* (Apse et al., 1999) and in tomato (Zhang and Blumwald, 2001).

2.4.2 Induced biosynthesis of compatible solutes and antioxidative enzymes

Compatible solutes accumulate to high concentrations without affecting intracellular physiology (Bohnert and Jensen, 1996). These compounds have a

minimal effect on the pH or charge balance of the cytosol. The compatible solutes include amino acids such as proline (Singh et al., 2000) and ecotine (Lippert and Galinski, 1992), quaternary amines such as glycine betaine (GB) (Rhodes and Hanson, 1993; Wang and Nii, 2000), sugars such as glucose and fructose (Kerepesi and Galiba, 2000; Bohnert and Jensen, 1996) and polyols such as mannitols (Ford, 1984; Popp et al., 1985; Orthen et al., 1994). These molecules do not inhibit or interfere with normal enzymatic activities even at relatively higher concentrations (Johnson et al., 1968; Hasegawa et al., 2000).

Proline, as an osmolyte, is widely distributed across various kingdoms and genera (Mc Cue and Hanson, 1990). It is an important compatible solute found in many halophytes as well as glycophytes (Stewart and Lee, 1974; McCree, 1986). Rice and alfalfa plants exposed to high salinity accumulated higher levels of proline than control plants (Lutts et al., 1999; Ginzberg et al., 1999). Similarly, wheat plants transformed with proline biosynthesis genes showed improved tolerance to salinity (Sawahel and Hassan, 2002). In durum wheat (*Triticum durum*), Mattioni et al. (1997) reported that the concentration of proline increased when exposed to salinity stress. Proline, glycine betaine and other sulphonium compounds are charge neutral (zwitterion) at physiological pH which make them ideal candidate for osmoprotection under stress conditions. Proline and most other osmolytes are localized in higher amounts in cytoplasm than in vacuoles (Aubert et al., 1999).

The concentration of glycine betaine (GB) increases under salinity stress in many plant species (Saneoka et al., 1999; Wang and Nii, 2000). Genetic transformat-

ions of plants with genes synthesizing GB have shown significant protection against salinity stress. *Arabidopsis* plants over-expressing *codA* gene, derived from bacteria, were shown to tolerate higher concentrations of NaCl (Hayashi et al., 1997). Similar transformations with GB synthesizing genes in Brassica, rice, tomatoes and sweet potato improved plant performance under salinity stress (Prasad et al., 2000; Mohanty et al., 2002; Zhou et al,. 2007).

Carbohydrates, such as sugars (reducing and nonreducing) and starch, accumulate under salt stress (Parida et al., 2002). Their major functions include osmoprotection, osmotic adjustment and radical scavenging. Salt stress leads to increase in concentration of reducing sugars (glucose, fructose), sucrose, and fructans in a number of plants (Khatkar and Kuhad, 2000). NaCl stress significantly increased soluble sugar, saccharides and starch content in the leaves of tomato plants (Khavari-Nejad and Mostofi, 1998). Trehalose, a non reducing sugar, was shown to have protective effects against desiccation. Over-expression of trehalose in rice protected plants from multiple abiotic stresses (Garg et al., 2002).

Salt stress in plants induces the production of Reactive Oxygen Species. ROS has diverse functions. It is involved in protective and signaling pathways. A basal level of ROS is required for such processes. ROS level depends on a delicate equilibrium between its production and scavenging. ROS damage due to such ionic imbalance are largely local initially but spread as time proceeds (Ahmad et al., 2010). Plants have developed an inclusive antioxidant defense to protect from damages due to ROS. The metalloenzyme superoxide dismutase (SOD)

converts $O_2^{\cdot -}$ (superoxide radical) to H_2O_2 and molecular oxygen (Hernandez et al., 1995). The H_2O_2 detoxification reaction is catalyzed by ascorbate specific peroxidase, which is present in high concentrations in chloroplast (Chen and Asada, 1989), through the ascorbate–glutathione cycle (Halliwell and Gutteridge, 1985; Asada, 1992).

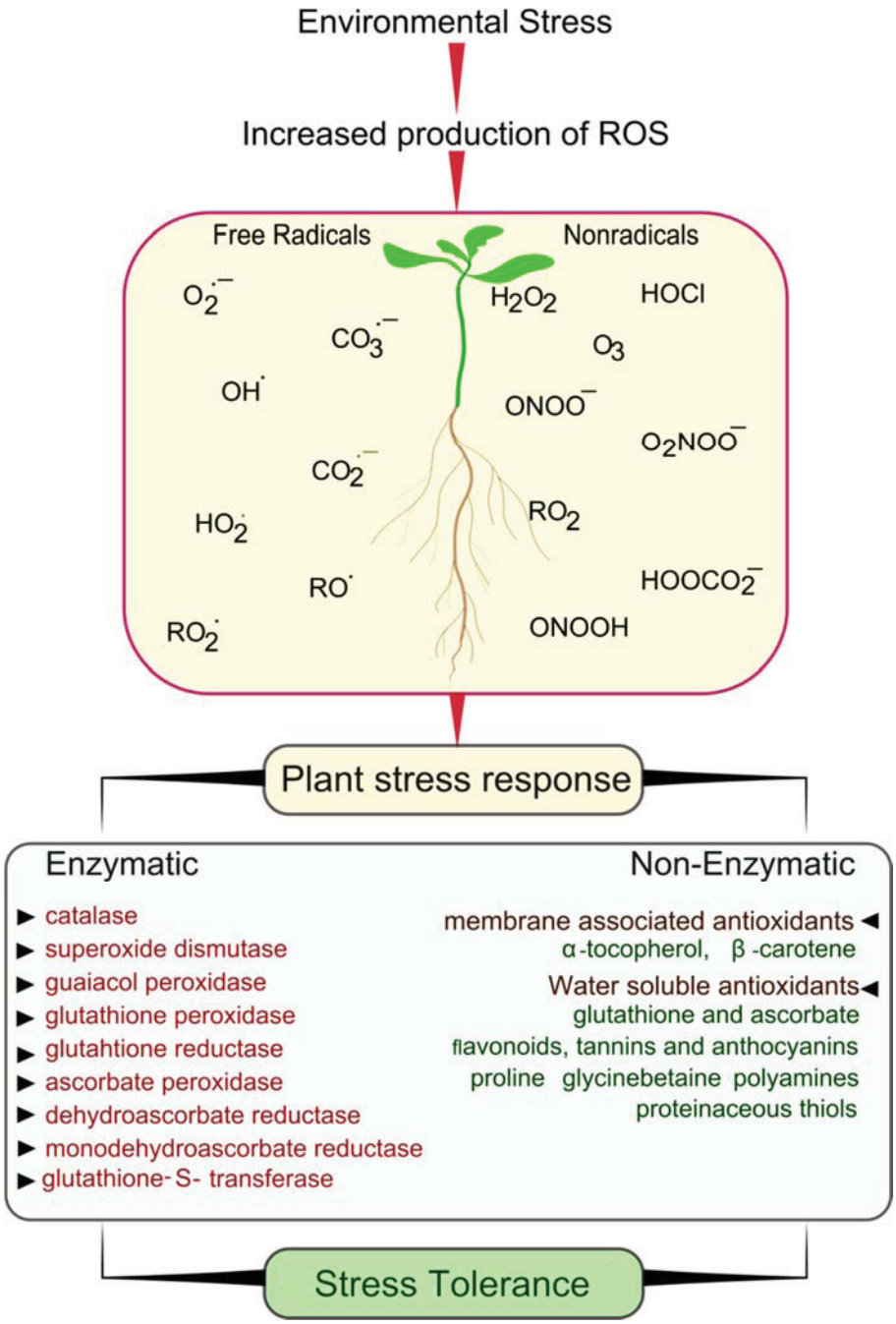


Figure 2.6: Response of plants to ROS

2.4.3 Role of plant hormones in stress tolerance

Plant hormones play a key role in regulating growth and development in plants. The major growth regulators are auxins, cytokinin (CK), gibberellin (GA), abscisic

acid (ABA), ethylene, jasmonic acid and brassinosteroids. Several physiological and developmental processes are under direct regulation of cytokinins. For example, cytokinins regulate cell division, chloroplast development and differentiation, and delay senescence. Cytokinin is also involved in nutrient mobilization (Mok and Mok, 2001). The role of ABA, ethylene, and jasmonates are pronounced mainly under biotic and abiotic stresses. Stresses limit plant growth and yield, and trigger early senescence. The involvement of abscisic acid (ABA) in salinity tolerance was described by Xiong et al. (2001). *Arabidopsis* mutants deficient in ABA were significantly affected by salinity stress. Popova et al. (1995) reported that ABA alleviated the inhibitory effect of NaCl on photosynthesis, growth and the translocation of assimilates. The concentrations of ABA and cytokinin increased under high salt concentration in *Mesembryanthemum crystallinum* and rice respectively (Thomas et al., 1992; Vaidyanathan et al., 1999). GomezCadenas et al. (1998 & 2002), reported increased levels of ABA and ethylene in salt stressed citrus plants, resulting in reduced injury due to toxic levels of Cl^- ions in the leaves. ABA regulates stomatal closure during water deficit periods and increases water use efficiency. ABA has been used in pretreatment or priming of seedlings to increase stress tolerance. Salt acclimation under gradual increase of salt concentrations is regulated by ABA (Noaman et al., 2002). Moreover, jasmonates, another group of plant hormone, also plays an important role in salt tolerance. Jasmonates mediate signaling, including activation of defense responses, flowering, and

senescence. Experimental evidence shows that salt tolerant tomato cultivars have higher levels of jasmonates than salt-sensitive cultivars (Hilda et al., 2003).

2.5 Recent approaches in mitigation of salinity stress in plants

Most crop plants are glycophytes and are not capable of growing in soil that has high concentrations of salts. Attempts to improve salt tolerance through conventional breeding, have met with limited success, primarily because salt tolerance is a multigenic trait (Flowers, 2004). Flowers and Yeo (1995) proposed five possible genetic procedures to improve crop productivity under saline conditions; i) deployment of halophytes as alternative crops, ii) exploitation of genetic variation for salt tolerance already present in existing crop species iii) use of interspecific hybridization to increase salt tolerance of current commercial cultivars, iv) generation of variation within existing crops via genetic mutation, and v) breeding for higher yield, rather than salt tolerance. All of these strategies are still under development and evaluation. Because of the present status of limited crop productivity under saline conditions, the development of new methodologies and strategies is essential. The application of seaweed and seaweed products has been documented to alleviate a wide range of stresses (Craigie, 2011). These useful seaweeds include *Ascophyllum nodosum,* which is discussed in **Section 2.6**.

2.6 Tomato, salinity and model for crop plant studies

Tomato (*Solanum lycopersicum* Mill.) is an important vegetable crop (Cantore et al., 2005). Tomato has also been widely used as a model system to study physiological and molecular basis of fruit development (The Tomato Genome Consortium, 2012). Tomato is consumed in a number of ways; ripe fruit is served fresh, cooked, or processed (canning, juice, pulp, paste, sauce or even dried). The cultivated tomato is adapted to different climates, though production is greatest in dry Mediterranean areas with adequate irrigation (Cuartero and Fernandez-Munoz, 1998). Tomato is moderately sensitive to salinity (Foolad, 2004). Low salinity levels reduce tomato seed germination rate and also lengthen the time needed to complete full germination (Ayers et al, 1952). Soils with electrical conductivities close to 4-6 dS/m limit optimum growth of tomato and EC above 6 dS/m significantly reduces root growth (Nanawati and Maliwal, 1974; Papadopoulos and Rendig, 1983). A strong inverse relationship was observed between EC and water uptake. High soil salinity led to non-competitive inhibition of the nitrate ions due to membrane depolarization (Cram, 1983; Suhayda et al., 1990; Hawkins and Lewis, 1993). Saline conditions decrease the relative proportions of other essential ions like K^+, Ca^{2+}, Mg^{2+} and NO_3^- and aggravate the toxic effects of Na^+ and Cl^- ions. Tomato plants are more susceptible to salinity at the seedling stage as compared to flowering and fruit development stages (Dumbroff and Cooper, 1974).

2.7 *Ascophyllum nodosum* (L.) Le Jol. and plant stress alleviation

Ascophyllum nodosum, a large brown alga, is perennial seaweed confined to the intertidal zone of the North Atlantic Ocean. A maximum temperature of these coastal zones is ~ 27 ˚C (Keser et al., 2005). Shoots of this seaweed arise from a holdfast and develop a complex structure of dichotomous lateral branches. Bladders are centrally located on long flattened strap-like fronds which hang down, draping intertidal rocks. Many fronds grow from the base and new fronds are regenerated from the base when the larger fronds are damaged (Ugarte and Sharp, 2001).

Kohlmeyer and Kohlmeyer (1972) proposed a lichenous relationship of *Ascophyllum nodosum* with an ascomycete, *Mycophycias ascophylli,* which has been intensively studied in the recent years. Interestingly, all *Ascophyllum* collected in nature are infected or associated with this fungus. No cellular invasion or penetration has been recorded on *Ascophyllum* by the fungus, allowing for an obligate and mutualistic symbiosis (Garbary and Gautam, 1989; Garbary and Deckert, 2001; Xu et al., 2008). This form of species interaction was termed as symbiotum by Deckert and Garbary (2005).

A. nodosum has been extensively used in agriculture as plant biostimulant (Craige, 2011). *Ascophyllum nodosum* extract (ANE), when applied to plants, stimulates shoot growth and branching (Temple and Bomke, 1989),increases lateral root development (Metting et al., 1990), and improves nutrient uptake (Yan, 1993). ANE has also been reported to improve plants' tolerance to environmental stresses such as drought, salinity and frost (Nabati, 1991; Nabati

et al., 1994). Application of *A. nodosum* extract has also been shown to impart stress tolerance in sensitive crop plants. Studies on citrus, grapes, Bermuda grass and Kentucky blue grass have demonstrated that ANE improved abiotic stress tolerance (Zhang, 1997; Zhang and Schmidt, 1999; Fike et al., 2001). Several bioactive compounds, including betaines (like γ-aminobutyric acid betaine, δ-aminovaleric acid betaine, laminine (N^6, N^6, N^6-trimethyl lysine), and glycine-betaine have been detected in *A. nodosum* and in the commercial products of *A. nodosum* (Blunden et al., 1985).

The goal of this research was to investigate the level of tolerance that *Ascophyllum nodosum* industrial organic extract (hereafter used as ANE) provides against salinity stress in tomatoes. The objectives of the research were: i) To determine the level of protection *A. nodosum* extracts (ANE) offer against salinity stress in tomato; ii) To study the biochemical basis of ANE mediated salinity tolerance in tomato; and iii) To elucidate the genetic basis of ANE mediated salinity tolerance in tomato by studying the differential regulation of some of the stress response genes, using quantitative polymerase chain reaction.

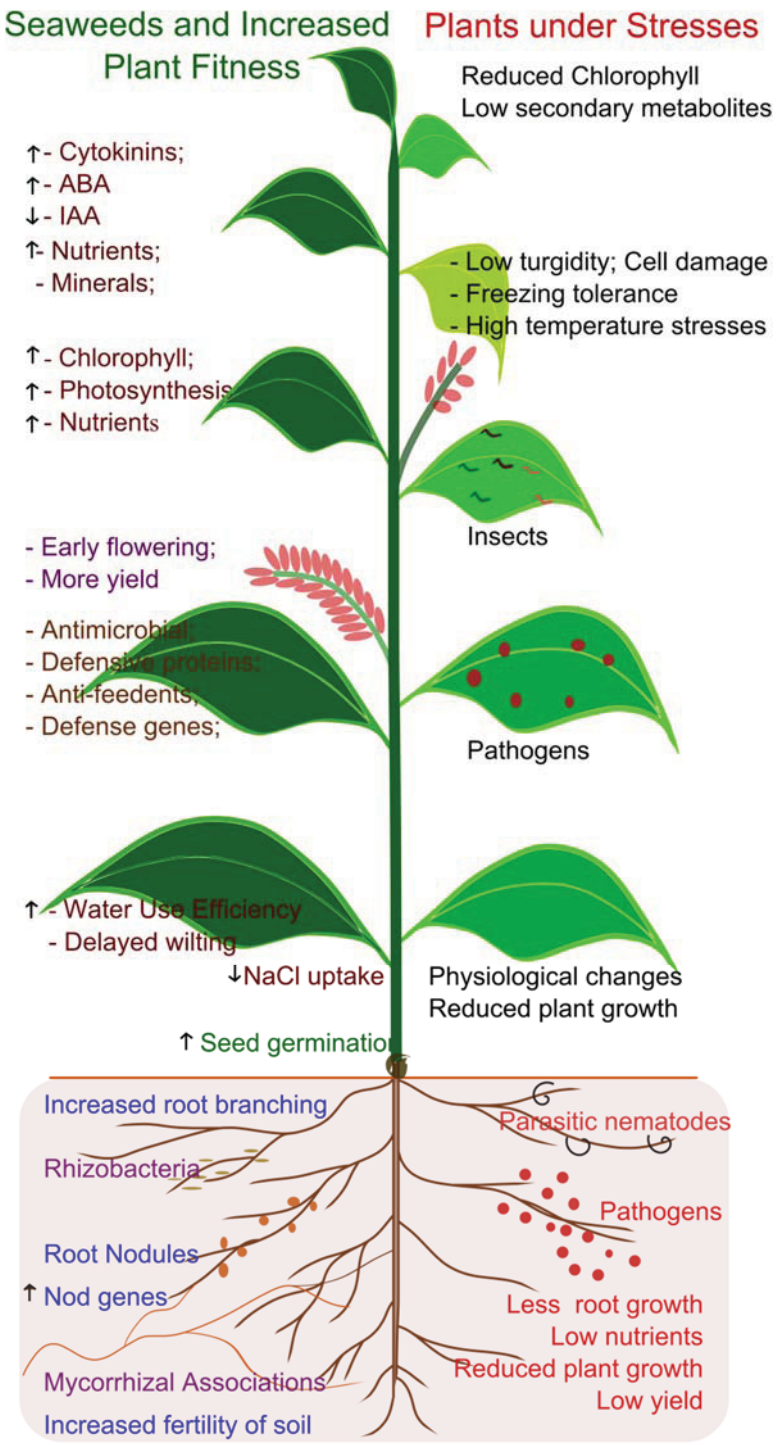


Figure 2.7: Effects of seaweed on plant fitness

CHAPTER 3 Material and methods

3.1 *In vitro* effect of ethyl acetate organic fraction of *Ascophyllum nodosum* on salinity tolerance in tomato seedlings

3.1.1 Preparation of organic sub fractions from *Ascophyllum nodosum* extract

All glassware used were washed and rinsed with 95% methanol. A methanol fraction of *Ascophyllum nodosum* extract (ANE) was obtained by suspending 20 g of solid ANE (water soluble concentrate), in 200 mL of absolute methanol, with occasional vigorous shaking by hand, for 10 minutes. The mixture obtained was filtered and dried under a continuous stream of N_2 and suspended in 50 mL of ultrapure Milli-Q® water. Subsequently, organic fractions were prepared by adding 150 mL of HPLC grade hexane (C_6H_{14}), chloroform ($CHCl_3$) and ethyl acetate ($C_4H_8O_2$) (Fisher Scientific) respectively to the methanol water extract. The three (hexane, chloroform and ethyl acetate) sub-fractions obtained were dried under a continuous stream of N_2, re-suspended in 10 mL of methanol and stored at −10 °C after every use **(Appendix 7.1)**. The ethyl acetate fraction of *Ascophyllum nodosum* extract (EtOAc-ANE) was used in all the *in vitro* experiments.

3.1.2 Tomato plant seedling establishment for *in vitro* experiments

Tomato seeds (variety- Scotia), were surface sterilized in a sterile 15 mL tube for 2 minutes in two volumes of 70% ethanol, decanted and followed by two volumes of commercial bleach for 10 minutes, with frequent vortexing. The seeds were washed five times with sterile distilled water to remove the bleach residues. Subsequently seeds were transferred on the plates containing half strength

Murashige and Skoog 1962 (MS) basal medium supplemented with 10 g/L sucrose and 3 g/L Gelzan as solidifying agent. The plates were incubated at room temperature in the dark. After germination (~3 days), seedlings were transferred to Petri dish containing half strength MS medium (Murashige and Skoog, 1962) and placed under fluorescent light (100 mol photons $m^{-2}s^{-1}$) with a 16:8 h photoperiod at 25 ± 2 °C for 4 days. Uniform plants (approximately 5 cm long), with well differentiated roots and shoots, were used in the experiment.

3.1.3 Experimental setup and plant phenotype data collection and analysis

The experiment was set up as a completely randomized design where each treatment was applied on three plants per replication. This experiment was repeated three times.

Treatments constituted two NaCl levels [No salt (control), 100 mM] and two levels of *Ascophyllum nodosum* extract [No ANE (methanolic control or MeOH), 1 g/L ~ANE ethyl acetate fraction (hereafter EtOAc)]. Half strength liquid MS medium with and without 100 mM NaCl and EtOAc-ANE, treatments were prepared (EtOAc-ANE fractions were added post autoclaving). Uniform seedlings were then aseptically transferred to 15 mL autoclaved glass test tubes containing 10 mL of half strength liquid MS medium containing respective treatments and placed on the shelves under cool fluorescent light (100 mol photons $m^{-2}s^{-1}$) with a 16:8 h photoperiod at 25 ± 2 °C. The water levels in the tubes were maintained to avoid changes in the concentration of the treatments due to evapotranspiration. Fourteen days later the plants were harvested to measure leaf area, root length and root area. The leaves were scanned using Epson

Expression 10000 XL (Epson Canada Ltd., Markham, ON. Canada) and the images were analyzed using WinRHIZO and WinFOLIA Software packages (Regent Instruments Canada Inc, 2008). The average leaf area, root length and root area were calculated for each plant.

Data was analyzed with a one way ANOVA using Proc MIXED procedure, with Statistical Analysis Software (SAS 9.3,SAS Institute, Cary, C, USA) at p= 0.05. The Tukey's honestly significant difference (HSD) post hoc test was performed in cases of significance (p= 0.05) to further separate the means per treatment.

3.2 Biochemical analysis of *Ascophyllum nodosum* induced salinity tolerance in tomato seedlings in *in vitro*

3.2.1 Crude enzyme extract preparation

A similar experimental setup was used as described in previous section. Leaf tissues (~ 1 g/ sampling time) collected at 0 h (before treatment application), 2, 6, 24 and 96 h were snap frozen in liquid nitrogen, freeze dried (Freezone 6, Labconco, Kansas, USA) and stored at -80 °C until use.

Crude enzyme extract was prepared from 10 mg lyophilized leaf tissue. The leaf samples were pulverized in a 2 mL microfuge tube with 3 mm silicon beads in a MINI-BEADBEATER™ (Biospec Products) for 20 seconds. 1 mL of cold enzyme extraction buffer [0.1 M KH_2PO_4 / K_2HPO_4 buffer (pH 7.5), 0.5% polyvinylpyrrolidone (PVP), and 3 mM Ethylenediaminetetraacetic acid (EDTA)] was added to the tubes and mixed well. The extracts were maintained at 4 °C on ice, throughout the experiment. The buffered samples were then centrifuged at

12,000 g for 20 min at 4 °C. The supernatant was pipetted into a clean 1.5 mL microfuge tube and was used for enzyme and biochemical analyses.

3.2.2 Estimation of total protein

The soluble protein in the crude enzyme extract was determined (Bradford, 1976) by reacting 40 µL of sample (5 µL of crude enzyme extract + 35 µL water) (section 2.2.2) with 200 µL Coomassie Plus-the Better Bradford™ Assay Kit (Pierce, Rockford, IL, USA) reagent in a 96 well microtiter plate (BioLite, Thermo Fisher Scientific). The bovine serum albumin (BSA) standards (125-2000 µg/mL) were loaded along with the samples and the absorbance at 595 nm was recorded using BioTek Power XS2 microplate reader (Bio-Tek, VT, USA) with Gen5™ software. Three technical replicates were performed and the amount of protein per sample was calculated using the BSA standard curve.

3.2.3 Estimation of catalase activity

The method described by Sarkar *et al.* (2009) was adapted with slight modification to fit microplate reader format. The catalase activity (CAT) was determined by reacting 5 µL of sample (1 µL of crude enzyme extract + 5 µL water) (section 2.2.2) with 200 µL 0.059 M hydrogen peroxide (BioShop® Canada Inc., Burlington, ON) in 0.05 M KH_2PO_4 / K_2HPO_4 buffer pH 7.0. The absorbance at 240 nm at intervals of 20 seconds for 2-3 minutes was recorded using BioTek Power XS2 microplate reader (VT, USA) with Gen5™ software. The rate of disappearance of H_2O_2 is followed by observing the rate of decrease in the absorbance at 240 nm. The change in absorbance OD240 /min from the initial linear portion of the curve was calculated. Thus, one unit of catalase will

decompose 1.0 µM of H_2O_2 per minute at pH 7.0 at 25 °C. Catalase activity was calculated using the following formula:

$$(\text{units/mg})\text{protein} = (\Delta\text{OD240/min})\ \text{X}\frac{1000}{43.6\ \text{X mg protein/mL reaction mix}}$$

3.2.4 Estimation of guaiacol peroxidase activity

Crude enzyme extracts were prepared as described in **Section 2.2.2**. A method described by Rahman and Punja (2005) was used with minor modifications. In brief, 0.05 M guaiacol solution was prepared in 25 mM sodium acetate buffer at pH 5.0. Crude extract (5 µL) was added to the 205 µL reaction mixture (200 µL + 5 µL) containing 8.8 mM hydrogen peroxide. The absorbance of the solution was recorded at 470 nm every 15 s for 2 min using a BioTek Power XS2 microplate reader (VT, USA) with Gen5™ software. All samples were run in triplicate. The change in the absorbance OD470 /min was calculated and the guaiacol peroxidase activity was expressed as OD470 /min/mg protein.

3.2.5 Estimation of proline

A modified microplate method (Carillo et al., 2011) was adopted. Lyophilized leaf tissue (~ 10 mg) was pulverized in a 2 mL microfuge tube followed by addition of 1 mL of 70% ethanol and vortexed. An aliquot of 200 µL of the ethanol extract was mixed with 400 µL of a reaction mixture containing ninhydrin 1% (w/v) in acetic acid 60% (v/v) and ethanol 20% (v/v). The tubes containing the reaction mixture were mixed, sealed and heated at 95 °C in a water bath for 20 minutes. The reaction was stopped at room temperature and an aliquot of 200 µL was run

in triplicate and read at 520 nm, using a BioTek Power XS2 microplate reader (VT, USA) with Gen5™ software. The proline content was expressed as:

$$\text{Proline}[\frac{\text{nmol}}{\text{mg Fresh Weight}}] = \frac{(\text{Abs extract} - \text{Abs blank})}{\text{Slope}} \text{x} \frac{\text{Volume of extract}}{\text{Volume of aliquot}} \text{x} \frac{1}{\text{Fresh Weight}}$$

3.2.6 Estimation of total chlorophyll

A modified DMSO (Dimethyl Sulphoxide) method of chlorophyll pigment extraction (Hiscox and Israelstam, 1978) was used. Lyophilized leaf tissue (~10 mg) was pulverized in 2 mL microfuge tube with 3 mm silicon beads in a MINI-BEADBEATER™ (Biospec Products) for 20 seconds. DMSO (1 mL) was added to the samples and kept on a heat block maintained at 65 °C for 5 minutes. The samples were centrifuged at 12000 *x g* for 5 minutes after which 200 µL of the supernatant was transferred to a microplate in triplicate (technical replicates). The absorbance was recorded at 663 nm and 645 nm using a BioTek Power XS2 microplate reader (VT, USA) with Gen5™ software. Chlorophyll a, b was calculated using the formula:

$$\text{chl } a \left(\frac{\text{mg}}{\text{g}}\right) = \{(12.7 \text{ X A663}) - (2.6 \text{ X A645})\} \text{ X } \frac{\text{mL of DMSO}}{\text{mg Leaf tissue}}$$

$$\text{chl } b \left(\frac{\text{mg}}{\text{g}}\right) = \{(22.9 \text{ X A645}) - (4.68 \text{ X A663})\} \text{ X } \frac{\text{mL of DMSO}}{\text{mg Leaf tissue}}$$

$$\text{Total Chl} = \text{Chl } a + \text{Chl } b$$

3.2.7 Estimation of Na^+ concentration

An alternative method was adopted to determine the change in the amount of sodium ion concentration in the growth medium (liquid half MS solution) after two

weeks of seedling growth. The seedlings were removed from their respective liquid half MS growth medium and were diluted with ultrapure Milli-Q® water to equal final volumes and used in atomic absorption unit to determine Na^+ concentration. The data were used to determine the amount of Na^+ absorbed by the tomato seedlings in two weeks.

3.3 Molecular analysis of *Ascophyllum nodosum* induced salinity tolerance in tomato seedlings *in vitro*

3.3.1 RNA isolation and cDNA synthesis

Total RNA was isolated using a modified single step TRIzol® method (Chomczynski and Sacchi, 1987). RNase free labware (tips, microfuge tubes) were used. Briefly, 10 mg (~100 mg fresh weight) of lyophilized leaf tissue was pulverized in a 2 mL microfuge tube with 3 mm silicon beads in a MINI-BEADBEATER™ (Biospec Products) for 20 seconds. TRIzol® (1 mL) was added to the tissue and kept for 5 minutes at room temperature. Subsequently, 200 µL of chloroform/ 1 mL of TRIzol® was added to the mixture and shaken vigorously for 15 seconds. The samples were centrifuged at 12000 *x g* for 15 minutes at 4 °C and the aqueous phase (supernatant) thus obtained was transferred to another microfuge tube by gentle pipetting, avoiding the interphase slurry. RNA was precipitated by adding 0.5 mL of 100% isopropanol per 1 mL of TRIzol® for 10 minutes and the samples were centrifuged at 12000 *x g* for 10 minutes at 4 °C. After decanting isopropanol, the pellet was washed with 1 mL of 75% ethanol and briefly centrifuged at 10000 *x g* for 5 minutes at 4 °C. Total RNA was suspended in RNase-free water and stored at -80 °C for further use.

RNA quantification was performed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) and the integrity of RNA was checked by visualizing the RNA bands on 1% agarose gel.

The RNA was treated with RQ1 DNAse (Promega Inc., USA) following the instructions provided by the manufacturer. The purified RNA thus obtained was reverse transcribed using a high capacity cDNA reverse transcript kit (Applied Biosystems, ON, Canada), according to manufacturer's instructions.

3.3.2 Quantitative Real Time PCR Analyses

A ten microliter reaction mixture was set up (cDNA, gene specific primers, 5 µL of 2X SYBR green reagent and 2.5 µL DEPC water) in a StepOne™ Real-Time PCR System (Applied Biosystems, CA). The *Actin* (*act*) gene was used to normalize transcript abundance. The following genes were used in this study; *Catalase-2, tompro-2, nhx-1* and *nhx-3.* The details of genes and the gene specific primer sets are presented in **Table 3.1.** The PCR conditions used were as specified in the manufacturer's instructions manual, including heat activation- 95 °C, 10 minutes, denaturation- 95 °C for 15 seconds, annealing and final extension at 60 °C for 1 minutes followed by 40 cycles. Relative transcript levels were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) which calculates the difference in transcript levels compared to control.

Table 3.1: Gene specific primers

Gene	Primers sequence	Gene description
catalase-2	F5'CCCAGAGGTATCGACTTGGA-3' R5'ATGAGCACACTTTGGAGCA-3'	hydrogen peroxide catabolic process
tompro-2	F5'AGTGATTACTTTTGGAGACAAGTCC-3' R5'GCAGCTTTTACTTTGGCAGTC-3'	Δ1-pyrroline-5-carboxylate synthetase
nhx-1	F5'CCGATGCACCTGAGTTGTC-3' R5'TGCTGCGTTTACACTGTCAATA-3'	Na^+/H^+ antiporter 1
nhx-3	F5'TGTCCAACTTGATGAAGAAGTCTC-3' R5'CACCATATTATGATTTGCTGGTTAAA-3'	Na^+/H^+ antiporter 3
act	F5'AATGTTCCTGCTATGTATGTTGCT-3' R5'AGTGGTACGACCGCTAGCAT-3'	Housekeeping gene (internal control)

3.4 The effect of commercial extract of *Ascophyllum nodosum* extract on salinity tolerance in tomato seedlings in greenhouse

3.4.1 Tomato plant seedling establishment for greenhouse experiments

Tomato seeds (variety- Scotia) were planted in small plug trays containing Promix BX (Premier Horticulture Inc, USA), watered and fertilized with 1 g/L of 20-20-20 NPK fertilizer (weekly). At the end of four weeks, seedlings were transplanted to larger pot containing Promix BX.

3.4.2 Experimental setup and plant phenotype data collection

Commercial *Ascophyllum nodosum* extract was provided by Acadian Seaplants Limited, Dartmouth, NS, Canada. The treatments constituted three different levels of ANE and sodium chloride.

The greenhouse experiment was conducted in a 3 x 3 'row plus column' design with three concentrations of salt [0.0 mM (control), 100 mM and 200 mM NaCl] levels on the rows and three *Ascophyllum nodosum* extract (ANE) [0.0 g/L (control), 0.3 g/L and 1 g/L] on the columns (John and Williams, 1995). The two factor experiment was replicated across six plantings. The pots were arranged in

the greenhouse at 25 °C and 16:8 h photoperiod. The 'row plus column' design was employed to evaluate the interaction between the salt and the ANE. A total of 54 uniform plants were used in one experiment (nine treatment combinations with six replicates). The treatment combinations were applied only once as a root drench only once during the experiment. The row and column randomizations were restricted to ensure all possible combinations. The plants' after treatment were allowed to grow for the next two weeks.

Figure 3.1: Experimental setup in the greenhouse

Plants were harvested and leaf area (cm^2), root length (cm) and root area (cm^2) were measured the using WinFOLIA and WinRHIZO software packages (Regent Instruments Inc., Sainte-Foy, Canada). The fresh weight (g) was also recorded. The experiment was repeated three times during the year 2011-2013.

a)

b)

Figure 3.2: a) Detached leaves for measurement of leaf area, **b)** Washed root for measurement of root length and root area

3.4.3 Determination of $Na^+$and K^+ concentration in leaf tissue of plants grown in greenhouse by atomic absorption spectrophotometry

All materials used were acid washed with 1 vol. HNO_3: 3 vol. H_2O to remove external traces of sodium metal contamination. Ultrapure Milli-Q® water was used throughout the procedure. Plant leaf samples were collected and dried in a hot air oven at 70 °C for 3 days and milled into 20 mesh sizes using a Wiley Mill (Arthur H. Thomas Company, PA, USA). The dried and powdered leaf samples were weighed (500 mg) and pre-ashed in evaporating dishes on an electric furnace for 3 minutes followed by complete ashing in a muffle furnace (Lindberg Hevi-Duty Electric, Watertown, Wisconsin) at 550 °C for 4 h. The ash were then dissolved in 20 mL of 0.6N HCl and filtered into a volumetric flask. The final volume was made to 100 mL with ultrapure Milli-Q® water and analyzed in an atomic absorption spectrometry unit. The amount of sodium was expressed as mg/g of dry weight of the leaf tissue.

3.5 Effect of EtOAc-ANE and commercial extract of *Ascophyllum nodosum* on tomato fruit number, yield and biomass in the greenhouse

3.5.1 Effect of EtOAc-ANE on tomato fruit number, yield and biomass

A greenhouse study was conducted to test the effect of EtOAc-ANE on tomato plant biomass, fruit number and yield. A completely randomized design was adopted and three treatments (control, 200 mM NaCl, EtOAc-ANE-200 mM NaCl) were used. Three replicates per treatment were used. The total number of fruits, fruit yield and aboveground fresh biomass of the plants were recorded after the plants stopped bearing new flowers and fruits. Data were analyzed with one way ANOVA using Proc MIXED procedure in SAS 9.3, (SAS Institute, Cary, C, USA) at p= 0.05 to determine significant difference between treatments.

3.5.2 Effect of commercial extract of *Ascophyllum nodosum* on tomato fruit number, yield and biomass

A completely randomized design was adopted. The treatments were Control, Long Ashton Nutrient Solution (LANS), ANE, 200 mM NaCl, LANS-200 mM NaCl, ANE-200 mM NaCl where LANS is the inorganic control to mimic the inorganic nutrient constitution of commercial ANE. Statistical analysis was performed as explained in previous section.

CHAPTER 4 Results

The ethyl acetate fraction of *Ascophyllum nodosum* extract (EtOAc-ANE) was used in the *in vitro* experiments while commercial *Ascophyllum nodosum* extract (ANE) (Acadian™, Acadian Seaplants Limited, Nova Scotia, Canada) was used in greenhouse experiments to understand the effect of *A. nodosum* extract on tomato plants under salinity.

Two week old tomato plants were used in *in vitro* experiments whereas four weeks old plants were used in greenhouse studies.

Biochemical and molecular analysis were only performed with leaf samples collected from the *in vitro* experiments.

LANS (Long Ashton Nutrient Solution) was used as an inorganic control during analysis of the sodium and potassium content of the leaf tissues as well as yield experiments with the commercial ANE.

4.1 *In vitro* experiments using ethyl acetate fraction of *Ascophyllum nodosum* extract

4.1.1 Effect of EtOAc-ANE on leaf area, root length and root area of tomato plants

Supplementation of 100 mM NaCl with EtOAc-ANE improved the growth and development of tomato plant. The treated plants recorded significantly higher ($p = 0.0034$) leaf area as compared to the 100 mM NaCl control (**Figure 4.1.1.1**). On an average, a ~25% increase in mean leaf area was observed in EtOAc- ANE-100 mM NaCl treated plants.

Similarly, the supplementation of 100 mM NaCl with EtOAc-ANE significantly (p= 0.001) increased the root length of the seedlings which was ~20% more than the plants treated with 100 mM NaCl (**Figure 4.1.1.2**).

The root surface area of 100 mM NaCl treated plants supplemented with EtOAc-ANE did not show a significant difference (**Figure 4.1.1.3**) with root surface area of non-supplemented plants.

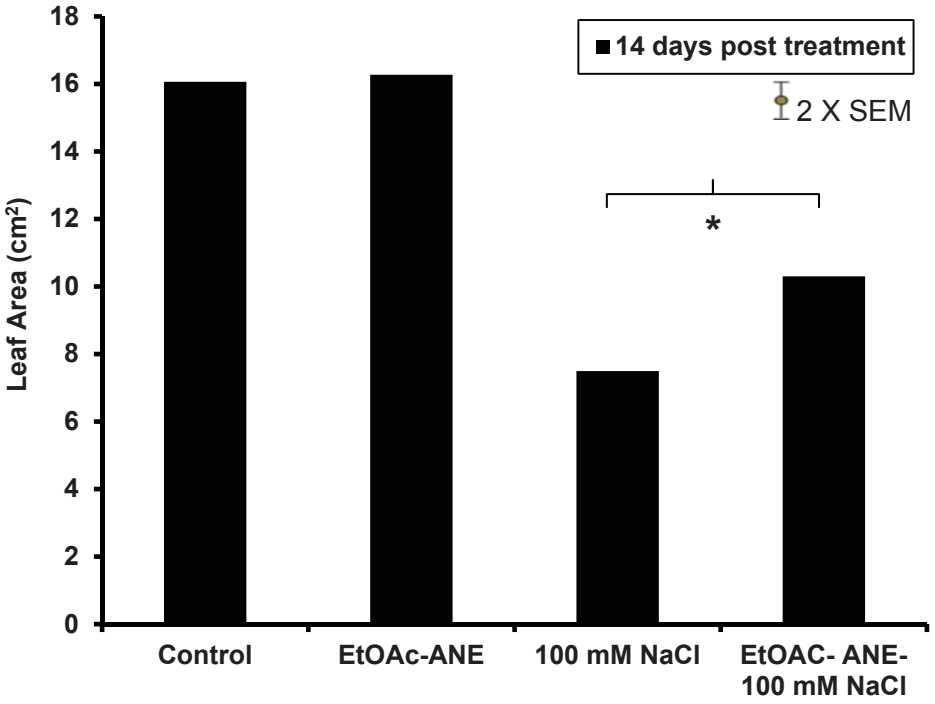


Figure 4.1.1.1 *Asocphyllum nodosum* induce salinity tolerance in *in vitro* grown tomato plants. The changes in leaf area of plants after 14 days, in 100 mM NaCl, with and without EtOAc-ANE supplementation are shown. Each value represents the average of samples collected from four plants and, 2X SEM (±0.506). Significantly different treatments are grouped. (* = $p < 0.05$).

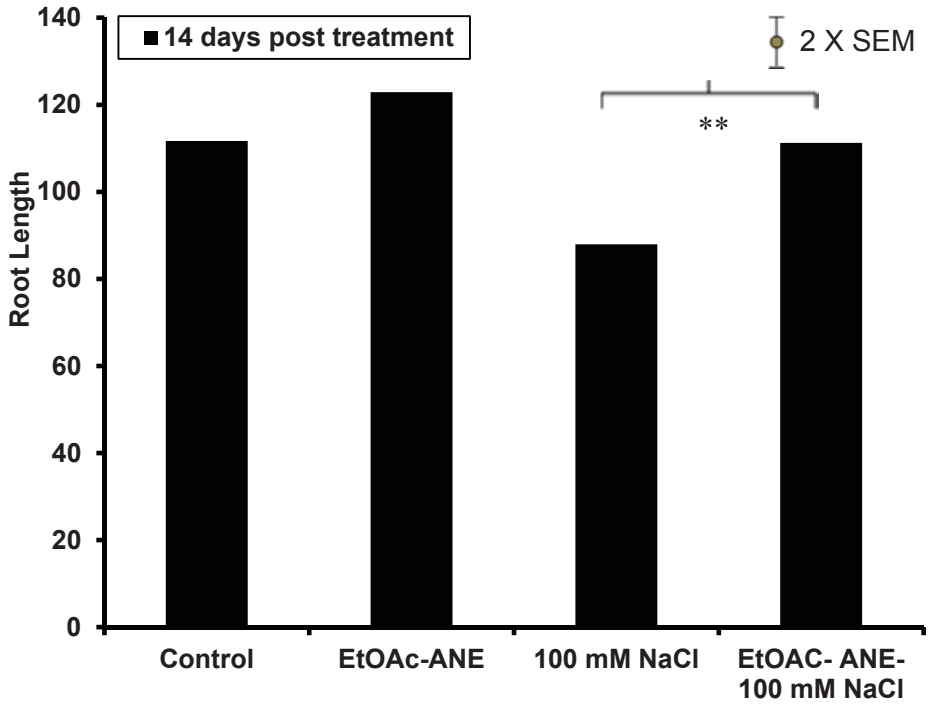


Figure 4.1.1.2 *Asocphyllum nodosum* induce salinity tolerance in *in vitro* grown tomato plants. The changes in root length of plants are after 14 days in 100 mM NaCl, with and without EtOAc-ANE supplementation are shown. Each value represents the average of samples collected from four plants and, 2X SEM (±3.191). Significantly different treatments are grouped. (** = $p < 0.01$).

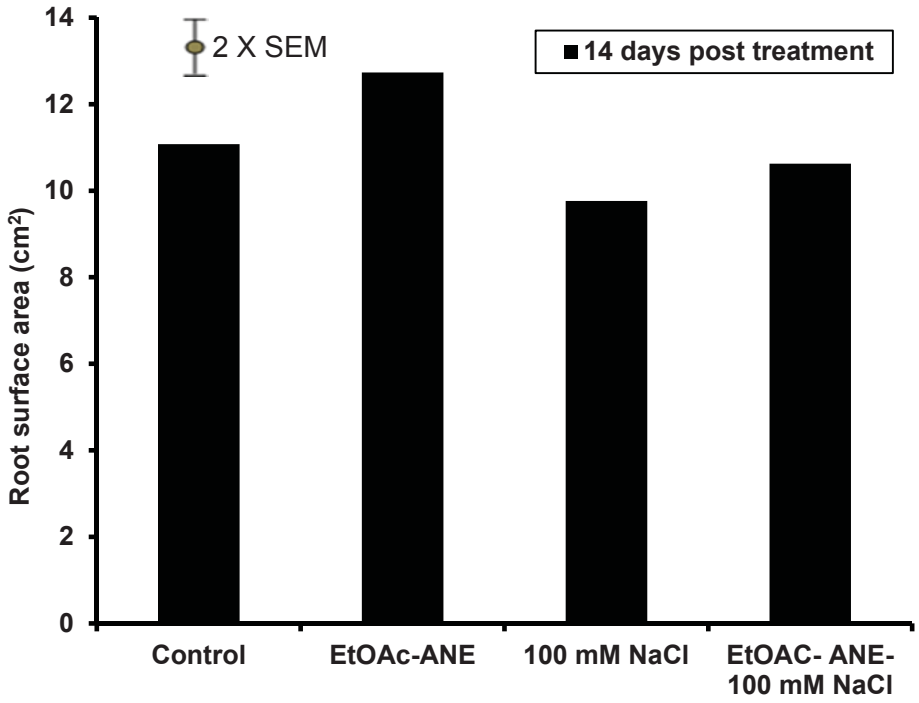


Figure 4.1.1.3 Changes in root surface area of plants after 14 days, in 100 mM NaCl, with and without EtOAc-ANE supplementation are shown. Each value represents the average of samples collected from four plants and, 2X SEM (±0.552).

4.1.2 Determination of biochemical changes in the leaves of tomato plants grown *in vitro* salinity stress studies

4.1.2.1 Estimation of catalase activity in the leaves of tomato plants

The catalase activity was meausred at 24 h and 96 h after the plants were treated with 100 mM NaCl, with and without EtOAc supplementation. The data was analysed in repeated measures in SAS 9.3, using PROC MIXED ($\alpha = 0.05$), followed by Tukey's (HSD) test. This was necessary to identify the effects due to treatment and time or their interactions (time x treatment). The catalase activity was 33% higher at 96 h post treatment in EtOAc-ANE-100 mM NaCl group. However, no significant difference was observed due to large variation within the groups (**Figure 4.1.2.1**). No significant difference was observed among 100 mM NaCl and EtOAc-ANE-100 mM NaCl groups between 24 h and 96 h. There was no significant effect of treatment or time, although a nearly significant interaction between the treatment and the time ($p= 0.0679$) was observed.

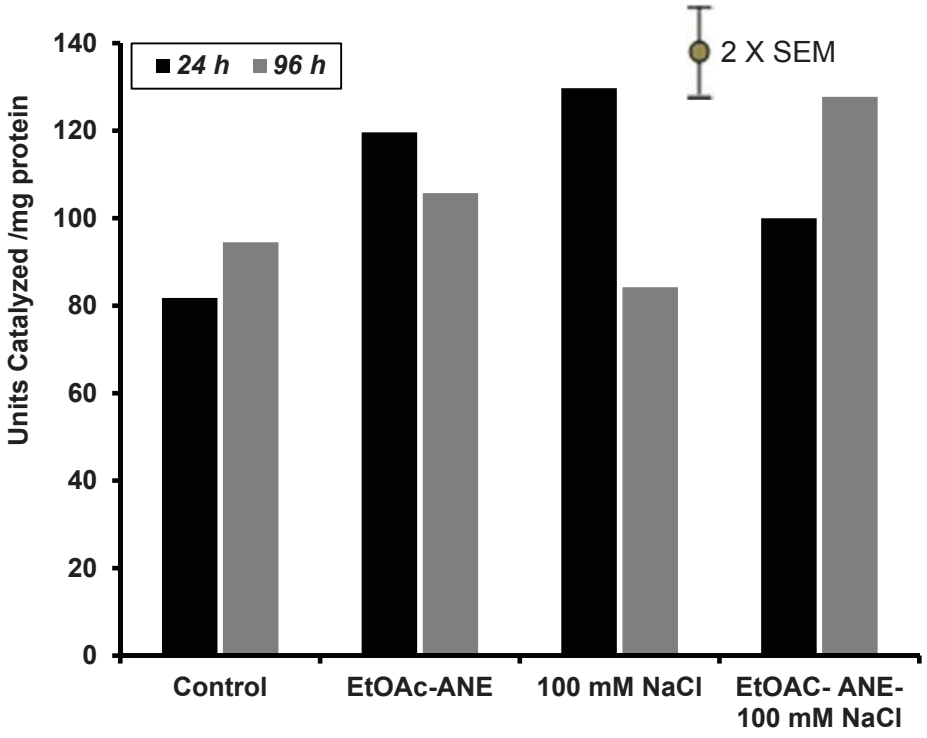


Figure 4.1.2.1 Changes in catalase activity of tomato leaves determined at 24 h and 96 h after treatments in 100 mM NaCl, with and without EtOAc-ANE supplementation. Each value represents the average of samples collected from three plants and, 2X SEM (±13.39).

4.1.3 Estimation of Guaiacol peroxidase activity in the leaves of tomato plants

The guaiacol peroxidase activity in the leaves was lower in EtOAc-ANE-100 mM NaCl treatments after 96 h (**Figure 4.1.2.2**). It was 41% lower when compared to 100 mM NaCl treatment but was not significant (p= 0.088) due to high variation in the sample means. The increase in guaiacol peroxidase activity in 100 mM NaCl treatment at 96 h was significantly different (p= 0.0131) from 24 h activity. The non-supplemeted 100 mM NaCl group showed 53% increase in guiacol peroxidase activity at 96 h compared to that at 24 h in the same group. The effects of treatment (p= 0.0091) and time (p= 0.0002) were highly significant,

although no significant interaction of time and treatment was observed (p= 0.2651).

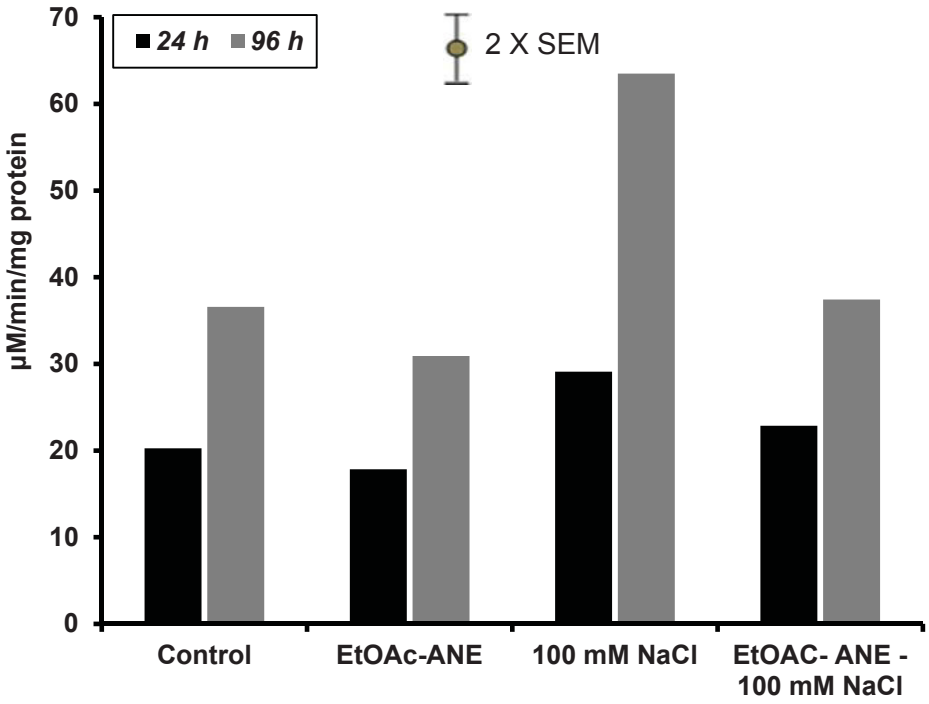


Figure 4.1.2.2 Changes in guaiacol peroxidase activity of the leaves of tomato plants determined at 24 h and 96 h following treatments in 100 mM NaCl, with and without EtOAc-ANE supplementation. Each value represents the average of samples collected from three plants and, 2X SEM (±5.84).

4.1.3.1 Estimation of proline content in the leaves of tomato plants

The proline content of the leaves significantly increased in 100 mM NaCl containing treatments after 96 h. The 100 mM NaCl supplemented with EtOAc-ANE showed a 15% increase over the non-supplemented 100 mM NaCl treatments (**Figure 4.1.2.3**) although not significant statistically due to large variance. Interestingly, at 24 h none of the treatments showed increased proline content.

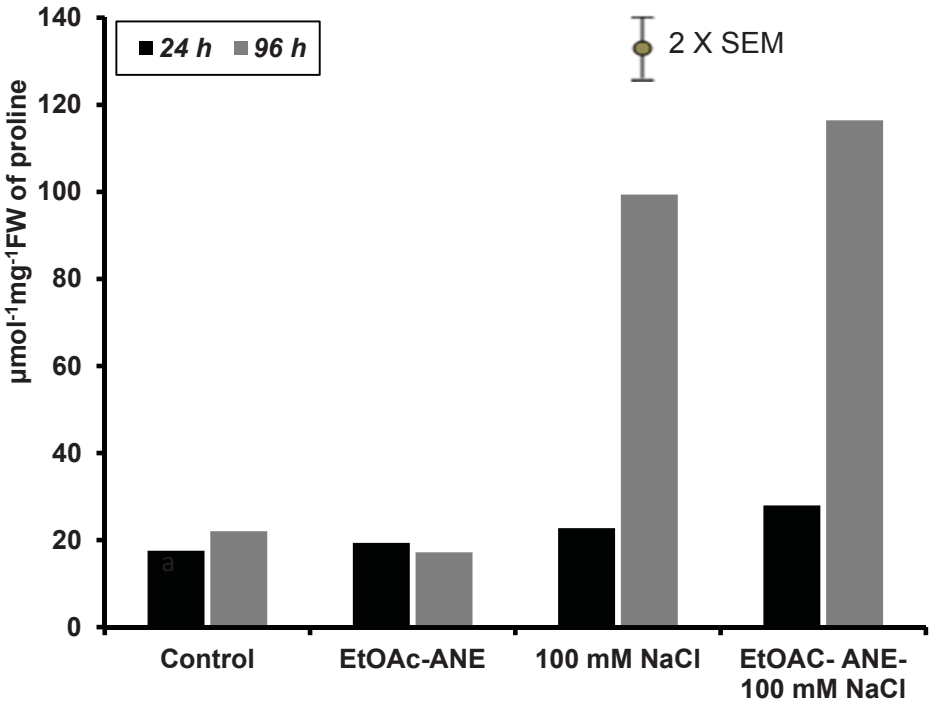


Figure 4.1.2.3 Changes in proline content of the leaves of tomato plants determined at 24 h and 96 h following treatments in 100 mM NaCl, with and without EtOAc-ANE supplementation. Each value represents the average of samples collected from three plants and, 2X SEM (±7.35).

4.1.3.2 Estimstion of MDA content in the leaves

The supplementation of 100 mM NaCl with EtOAc-ANE extract did not have effects on MDA concentrations of the leaves at 24 h and 96 h (**Figure 4.1.2.4**). The repeated measure analysis revealed only an expected significant difference between the treatments (p= 0.0252) and not for the time or time X treatment interaction. The higher level of MDA content in both 100 mM NaCl and EtOAc-ANE supplemented treatments suggests a direct effect of NaCl induced membrane damage.

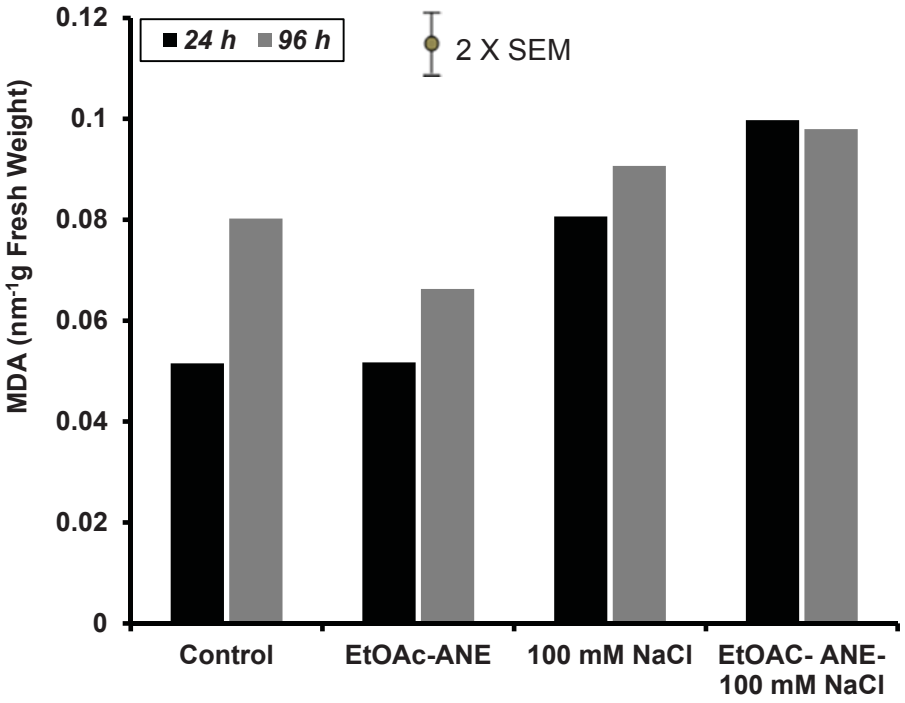


Figure 4.1.2.4 Changes in MDA (malondialdehyde) content of the leaves of tomato plants determined at 24 h and 96 h following treatments in 100 mM NaCl, with and without EtOAc-ANE supplementation. Each value represents the average of samples collected from three plants and, 2X SEM (±0.0128).

4.1.4 Estimation of chlorophyll content in the leaves

4.1.4.1 Estimation of chlorophyll *a* in the leaves

The Chlorophyll *a* content was significantly (p =0.030) higher (~25%) in 100 mM NaCl treatment as compared to EtOAc-ANE treatment at 24 h. However, a significant decrease (p= 0.015) in chlorophyll *a* was observed at 96 h in non supplemented 100 mM NaCl treatments **(Figure 4.1.3.1**). The EtOAc-ANE- 100 mM NaCl at 96 h showed a significant (p= 0.029) increase over the 100 mM NaCl treated group at 96 h. No significant decrease in chlorophyll *a* content was recorded in EtOAc-ANE supplemented 100 mM NaCl treatments at 96 h.

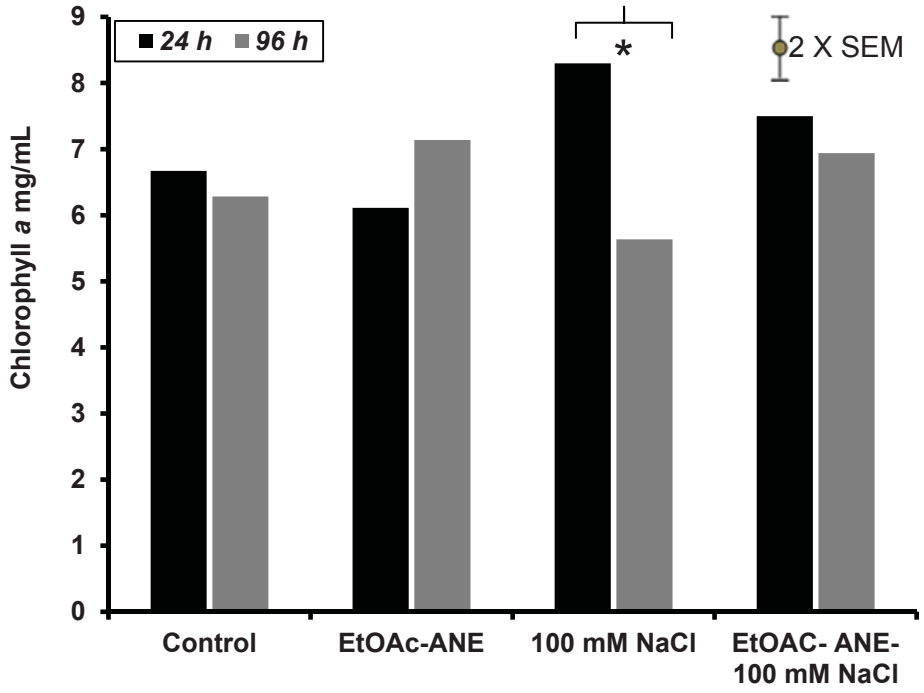


Figure 4.1.3.1 *Asocphyllum nodosum* induce salinity tolerance in *in vitro* grown tomato plants. Changes in chlorophyll *a* content of the leaves of tomato plants determined at 24 h and 96 h following treatments in 100 mM NaCl, with and without EtOAc-ANE supplementation. Each value represents the average of samples collected from three plants and, 2X SEM (±0.47). Significantly different treatments are grouped. (* = $p < 0.05$).

4.1.4.2 Estimation of chlorophyll *b* in the leaves

The change in chlorophyll *b* content in 100 mM NaCl treatment at 24 h and 96 h was significant (p= 0.0058) and larger than the decrease in chlorophyll *a* content (36%), at similar time points, as shown in **Figure 4.1.3.2**. The increase in chlorophyll *b* content at 96 h between 100 mM NaCl and EtOAc-ANE supplemented 100 mM NaCl group showed a weak statistical significance at p= 0.079.

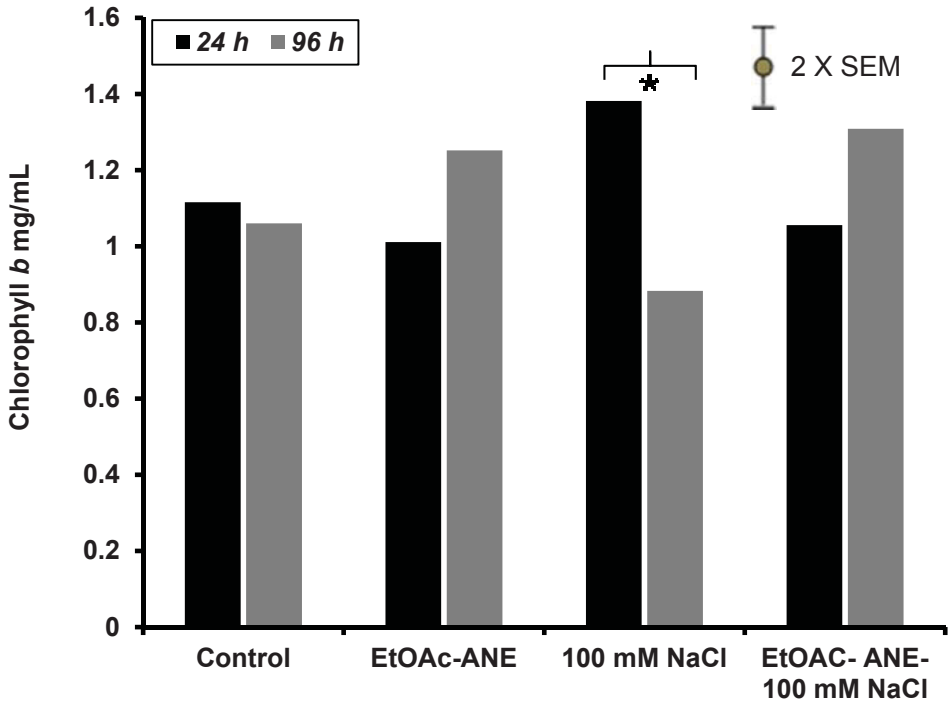


Figure 4.1.3.2 *Asocphyllum nodosum* induce salinity tolerance in *in vitro* grown tomato plants.Changes in chlorophyll *b* content of the leaves of tomato plants determined at 24 h and 96 h following treatments in 100 mM NaCl, with and without EtOAc-ANE supplementation. Each value represents the average of samples collected from three plants and, 2X SEM (±0.159). (* = $p < 0.05$).

4.1.4.3 Chlorophyll *a/b* Ratio

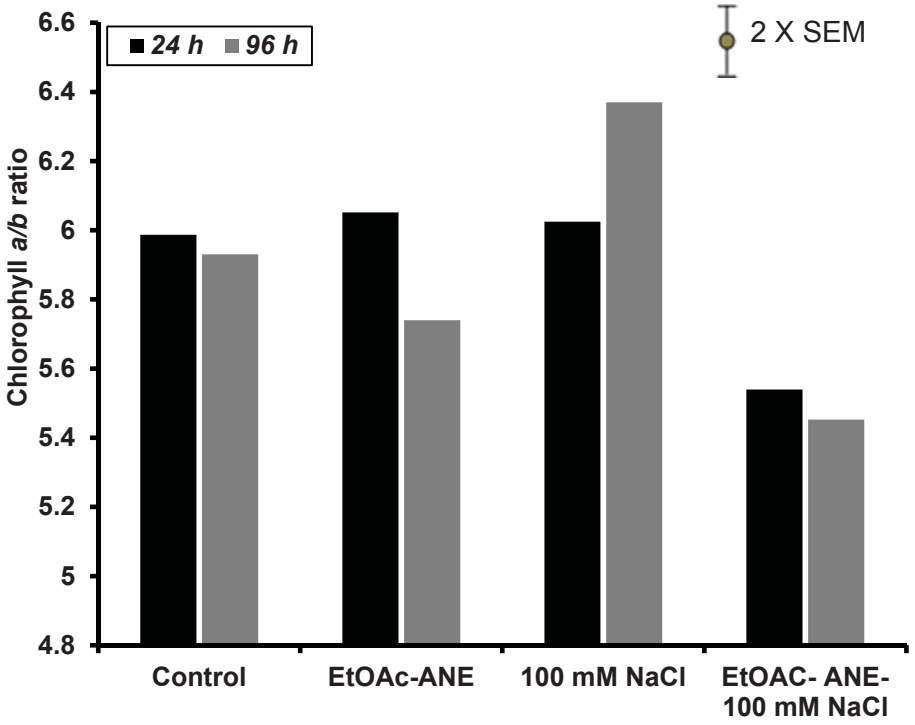


Figure 4.1.3.3 Changes in Chlorophyll *a/b* ratio of the leaves of tomato plants determined at 24 h and 96 h following treatments in 100 mM NaCl, with and without EtOAc-ANE supplementation. Each value represents the average of samples collected from three plants and, 2XSEM (±0.4287).

The chlorophyll *a/b* ratio was consistent among the groups, ranging from 5-7. No significant difference was observed as effects of time, treatment or their interactions.

4.1.4.4 Estimation of carotenoids in the leaves

A significant decrease (p=0.019) was recorded in non-supplemented 100 mM treatment groups from 24 h to 96 h post treatment. No differences were recorded between the treatments at 96 h (**Figure 4.1.3.4**). The EtOAC- ANE treatments did not show any difference in carotenoid content, irrespective of the treatment and the duration of the treatment. Interestingly, the fixed effects of time (p= 0.0263) and treatment X time (p= 0.0321) were significant which shows that the

carotenoid content was significantly different between at least one treatment group.

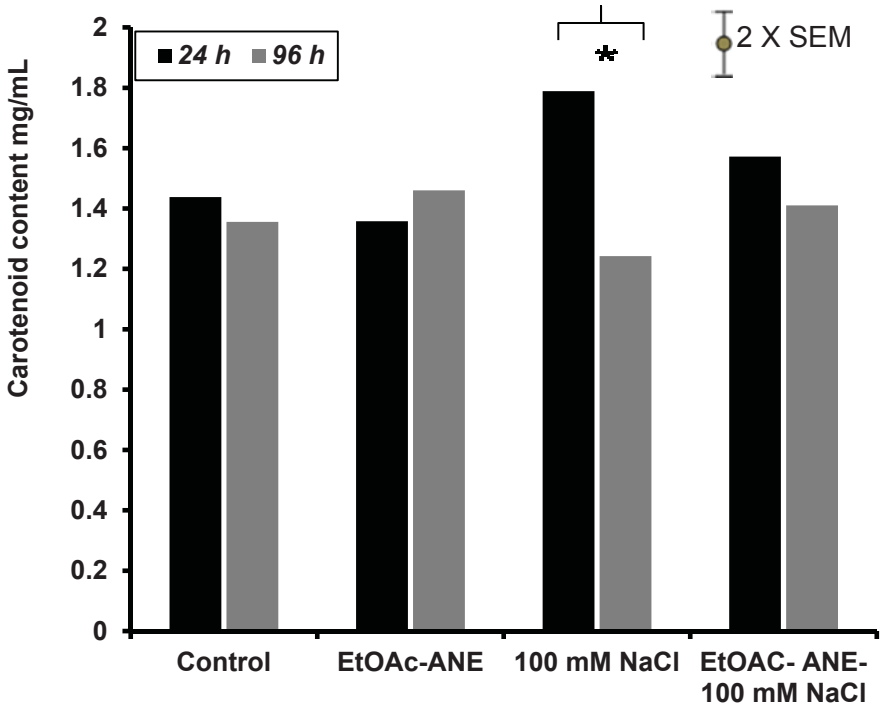


Figure 4.1.3.4 *Asocphyllum nodosum* induce salinity tolerance in *in vitro* grown tomato plants. Changes in carotenoid content of the leaves of tomato plants determined at 24 h and 96 h following treatments in 100 mM NaCl, with and without EtOAc-ANE supplementation. Each value represents the average of samples collected from three plants and, 2X SEM (±0.099).

4.1.5 Estimation of Na^+ concentration in *in vitro* growth medium

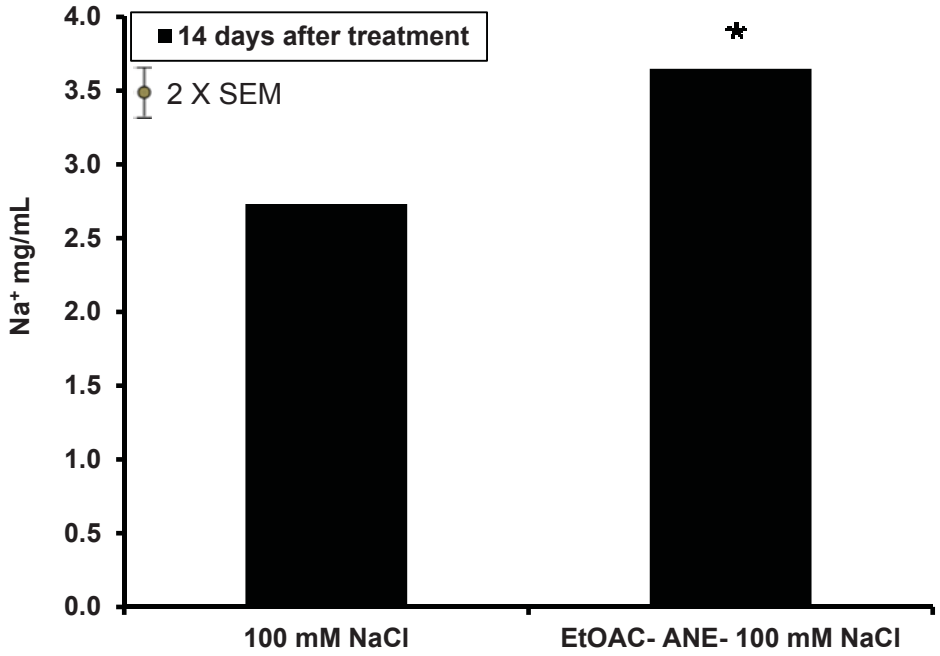


Figure 4.1.4 Effect of EtOAc-ANE induces salinity tolerance in *in vitro* grown tomato plants by regulating Na^+ entry. The liquid 1/2MS growth medium was collected at the end of two weeks and was analyzed for its Na^+ content. Changes in Na^+ in 100 mM NaCl, with and without EtOAc-ANE supplementation are shown. Each value represents the average six samples, 2X SEM (±0.201). (* = $p < 0.05$).

The changes in the amount of sodium absorption by the plants was analyzed by finding the decrease in the residual sodium content in the growth medium at the end of the experiment. The residual Na^+ in the 1/2MS growth medium after two weeks was significantly more ($p= 0.009$) in the EtOAc- ANE- 100 mM supplemented treatments than in the 100 mM NaCl, which shows that EtOAc-ANE supplemented samples absorbed significantly less sodium compared to 100 mM NaCl treatments (**Figure 4.1.4**).

4.1.6 Molecular analysis of *Ascophyllum nodosum* extract induced salinity tolerance

The expression of key genes associated with salinity tolerance such as *catalase, tompro2, nhx-1* and *nhx-3* were investigated at two different time intervals of 24 h and 96 h after exposure to treatment.

The relative changes in the transcript abundance at 24 h were similar in all the treatments. There was ~ 0.65-fold downregulation of transcripts of the *catalase-2* gene at 96 h in both 100 mM NaCl and 100 mM NaCl-EtOAc-ANE treatments **(Figure 4.1.5.1)**

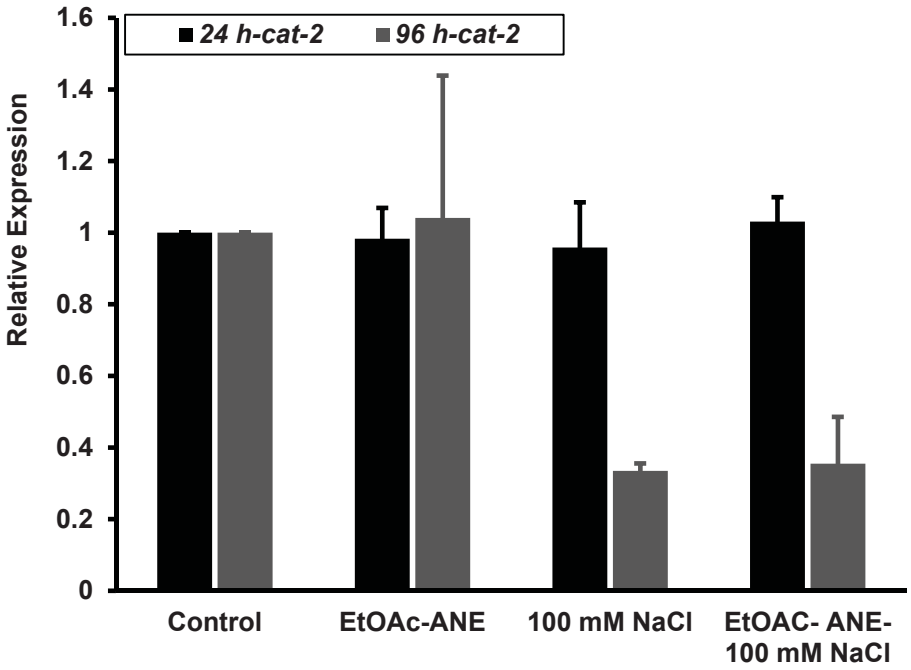


Figure 4.1.5.1 Changes in *catalase-2* gene expression in the leaves of tomato plants determined at 24 h and 96 h following treatments in 100 mM NaCl, with and without EtOAc-ANE supplementation. Each value represents the average of two biological replicates and vertical bars represent 2XSE of individual mean within the specific treatment.

The *tompro-2* gene exhibited a 1.25-fold increase (**Figure 4.1.5.2)** in the transcript abundance in 100 mM NaCl treatment at 24 h. All treatments expcept 100 mM NaCl showed constant transcript abundance at 24 h and 96 h.

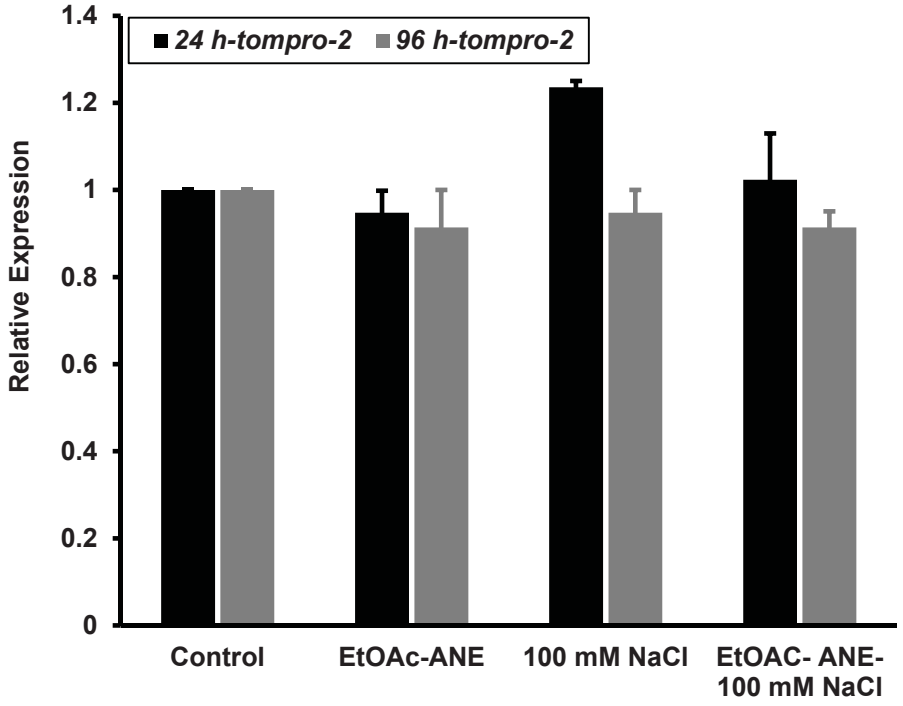


Figure 4.1.5.2 Changes in *tompro-2* gene expression in the leaves of tomato plants determined at 24 h and 96 h following treatments in 100 mM NaCl, with and without EtOAc-ANE supplementation. Each value represents the average of two biological replicates and vertical bars represent 2XSE of individual mean within the specific treatment.

The transcript abundance of the *nhx-1* gene increased 3.5- and 4.3-fold (**Figure 4.1.5.3)** in 100 mM NaCl and 100 mM NaCl-EtOAc-ANE treatments respectively after 24 h. Interestingly, the *nhx-1* gene activity was downregulated by 0.5-fold in control EtOAc-ANE treatment after 96 h. The transcript abundance of the gene returned to normal activity after 96 h in all the treatments.

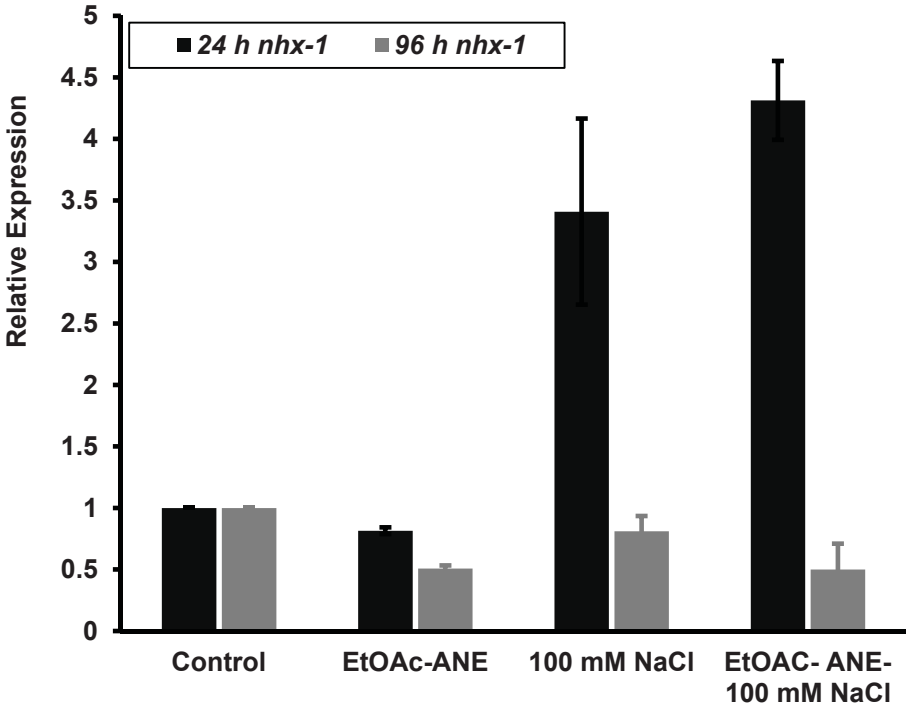


Figure 4.1.5.3 Changes in *nhx-1* gene expression in the leaves of tomato plants determined at 24 h and 96 h following treatments in 100 mM NaCl, with and without EtOAc-ANE supplementation. Each value represents the average of two biological replicates and vertical bars represent 2XSE of individual mean within the specific treatment.

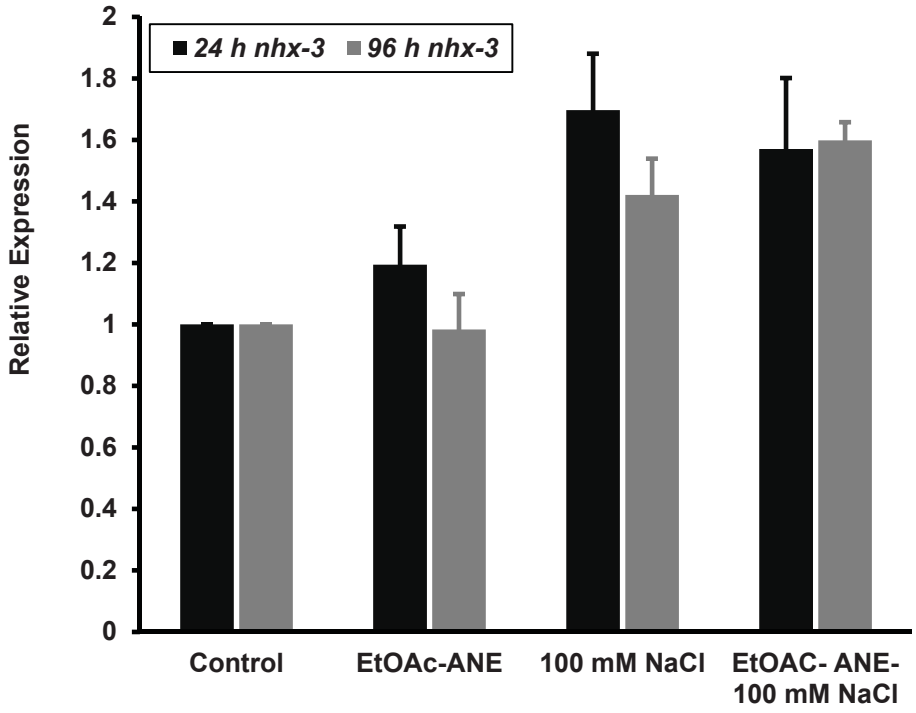


Figure 4.1.5.4 Changes in *nhx-3* gene expression in the leaves of tomato plants determined at 24 h and 96 h following treatments in 100 mM NaCl, with and without EtOAc-ANE supplementation. Each value represents the average of two biological replicates and vertical bars represent 2XSE of individual mean within the specific treatment.

The relative change in the transcript abundance of *nhx-3* gene was 1.5-fold in 100 mM NaCl containing treatments, at both the time points. The expression of the *nhx-3* gene was not influenced by the time and the gene expression was similar at 24 h and 96 h of treatment.

4.2 Greenhouse experiments with commercial *Ascophyllum nodosum* extract (ANE)

4.2.1 Effect of commercial *Ascophyllum nodosum* extract on leaf area, root length, root area and fresh weight of tomato plant

The two factor experiment replicated across six plantings in a 'row plus column' design was able detect a significant quadratic relationship (**Figure 4.2.1.1b**) (p= 0.005) of ANE with linear relationship (**Figure 4.2.1.1a**) obtained across NaCl

treatments. NaCl treatments showed significant differences with increasing concentrations of NaCl (p= 0.001). No linear effects were found with increasing concentrations of ANE. A decrease of 11% and 20% in the total leaf area was recorded when the salinity levels increased from 100 to 200 mM NaCl. No significant increase in the measured parameter was observed at similar levels of treatment controls or combinations of 0 mM, 100 mM and 200 mM of NaCl with 0 g/L, 0.3 g/L and 1.0 g/L of ANE.

A significant negative linear relationship (**Figure 4.2.1.2a**) was detected due to the reduction in the root length as salt concentration increased. No positive linearity was observed with increased concentrations of ANE. No significant increase was observed at similar levels of treatment control or combinations of 0 mM, 100 mM and 200 mM of NaCl with 0 g/L, 0.3 g/L and 1.0 g/L of ANE.

The decrease in the root surface area was similar to root length with a negative linear relationship (**Figure 4.2.1.3a**). No significant increase was observed at similar levels of treatment control or combinations of 0 mM, 100 mM and 200 mM of NaCl with 0 g/L, 0.3 g/L and 1.0 g/L of ANE. Similarly no significant increase in the fresh weight of the plants was observed at similar level of treatment controls and combinations of 0 mM, 100 mM and 200 mM of NaCl with 0 g/L, 0.3 g/L and 1.0 g/L of ANE (**Figure 4.2.1.4a**).

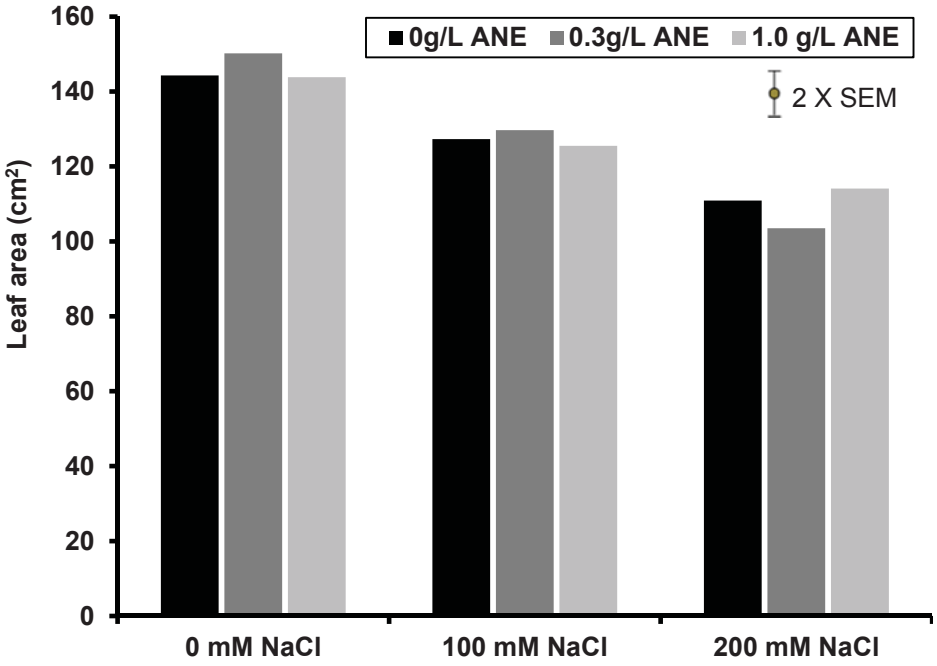


Figure 4.2.1.1: Changes in leaf area of tomato plants determined after 2 weeks of treatments with NaCl and commercial *Ascophyllum nodosum* extract in greenhouse. Each value represents the average of samples collected from six plants, 2X SEM (±2.719). Treatment combinations of 0 mM, 100 mM and 200 mM of NaCl with 0 g/L, 0.3 g/L and 1.0 g/L were respectively compared.

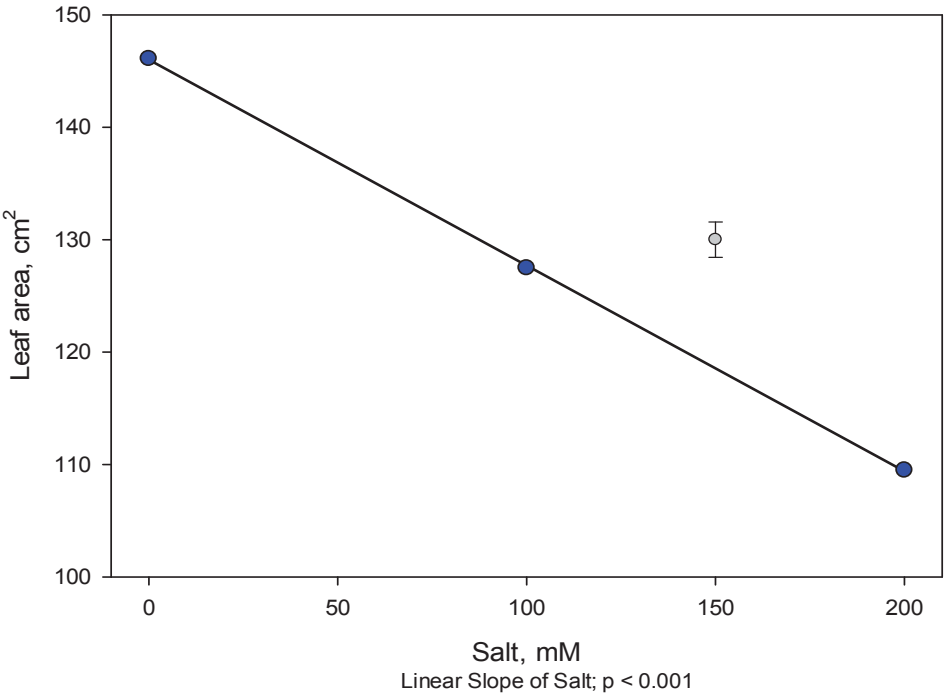


Figure 4.2.1.1a: Linear slope of NaCl, concentrations and its effect on leaf area at p<0.001. Vertical bar represents two standard errors of the mean (± SEM)

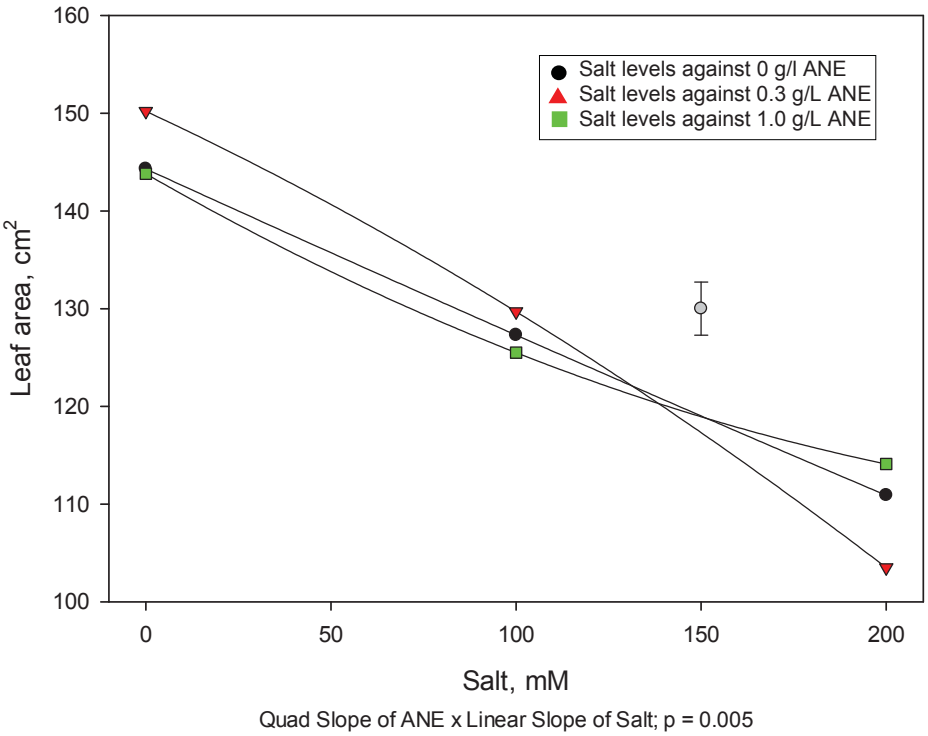


Figure 4.2.1.1b: Interaction of quadratic slope of ANE and linear slope of Salt at different levels on leaf area (p= 0.005). Vertical bar represents two standard errors of the mean (± SEM).

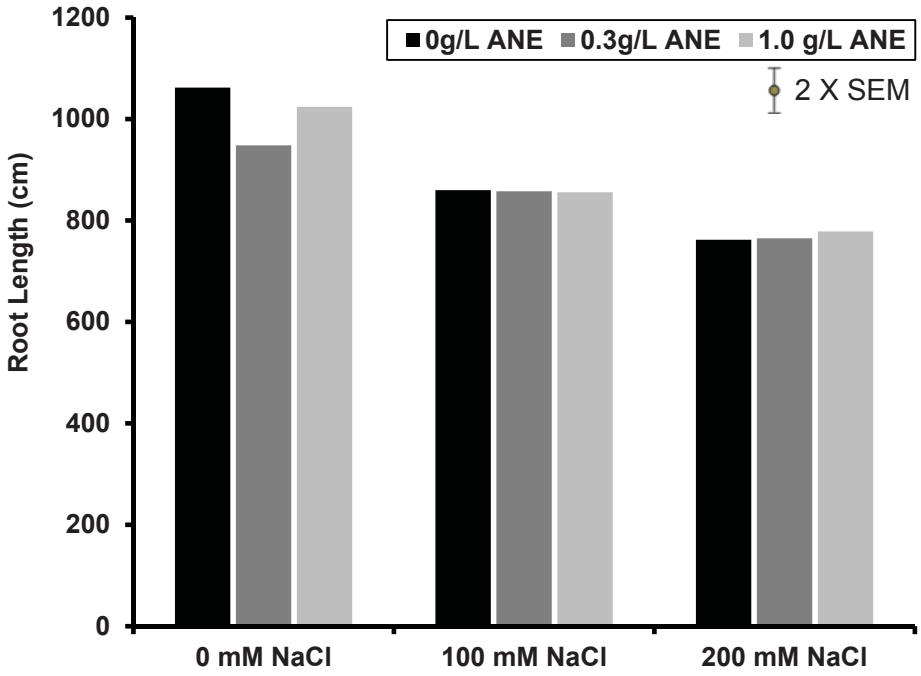


Figure 4.2.1.2: Changes in root length of tomato plants determined after 2 weeks of treatments with NaCl and commercial *Ascophyllum nodosum* extract in greenhouse. Each value represents the average of samples collected from six plants, 2X SEM (±35.56). Treatment combinations of 0 mM, 100 mM and 200 mM of NaCl with 0 g/L, 0.3 g/L and 1.0 g/L were respectively compared.

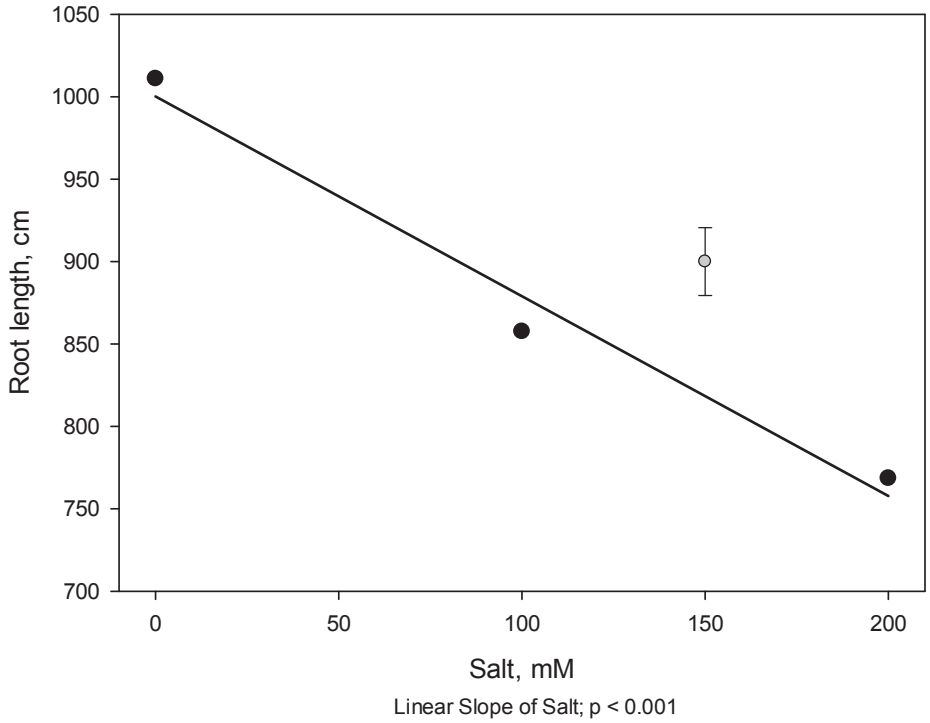


Figure 4.2.1.2a: Linear slope of NaCl concentrations and its effect on root length (p<0.001). Vertical bar represents two standard errors of the mean (± SEM)

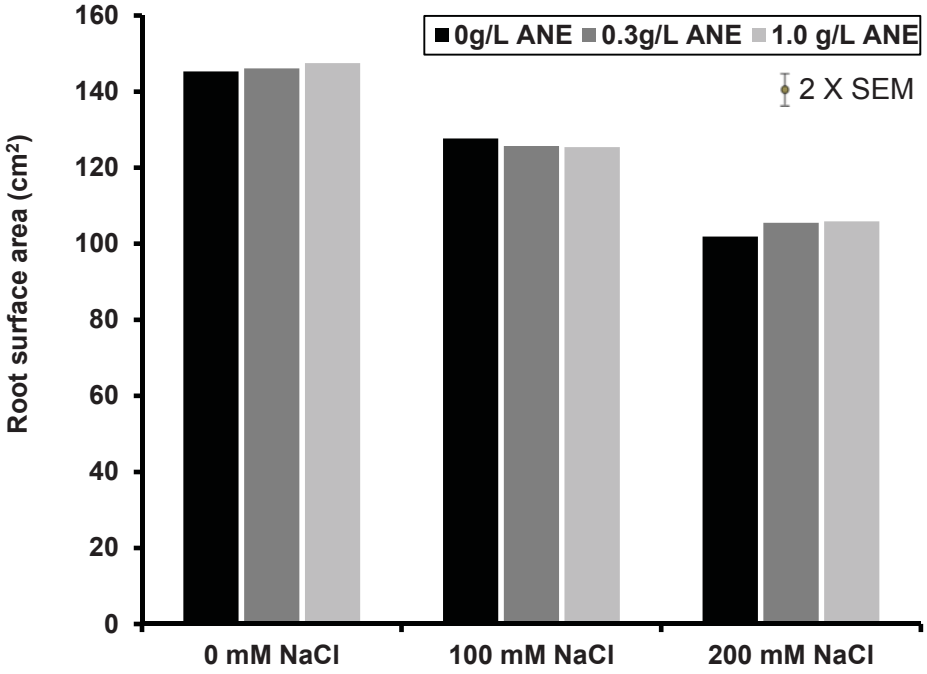


Figure 4.2.1.3: Changes in root surface area of tomato plants determined after 2 weeks of treatments with NaCl and commercial *Ascophyllum nodosum* extract in greenhouse. Each value represents the average of samples collected from six plants, 2X SEM (±2.635). Treatment combinations of 0 mM, 100 mM and 200 mM of NaCl with 0 g/L, 0.3 g/L and 1.0 g/L were respectively compared.

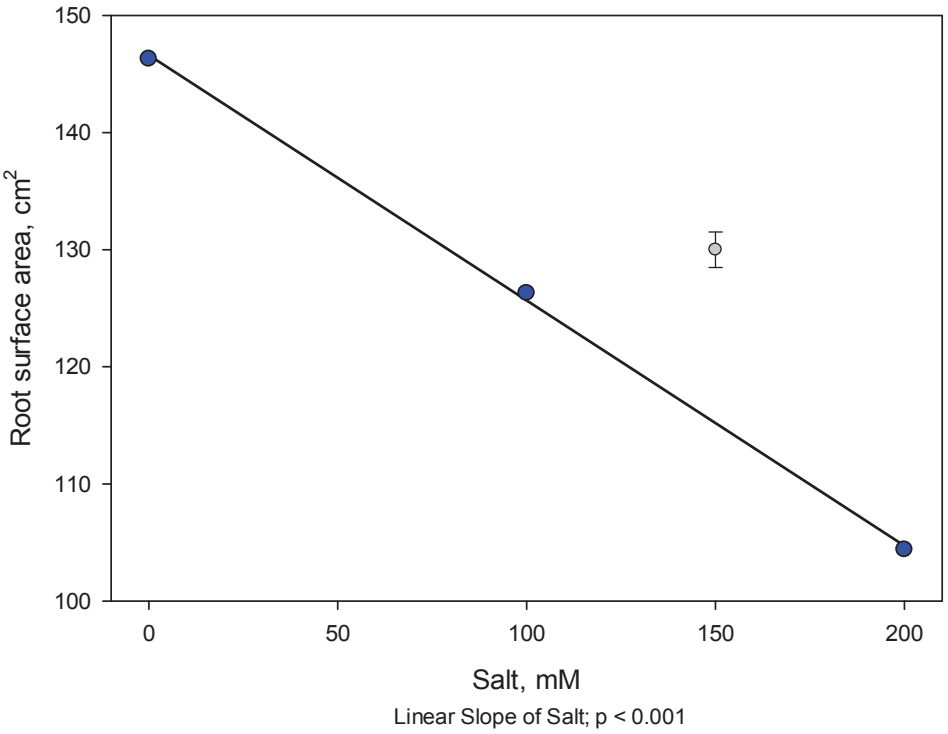


Figure 4.2.1.3a: Linear slope of NaCl concentrations and its effect on root length (p<0.001). Vertical bar represents two standard errors of the mean (± SEM)

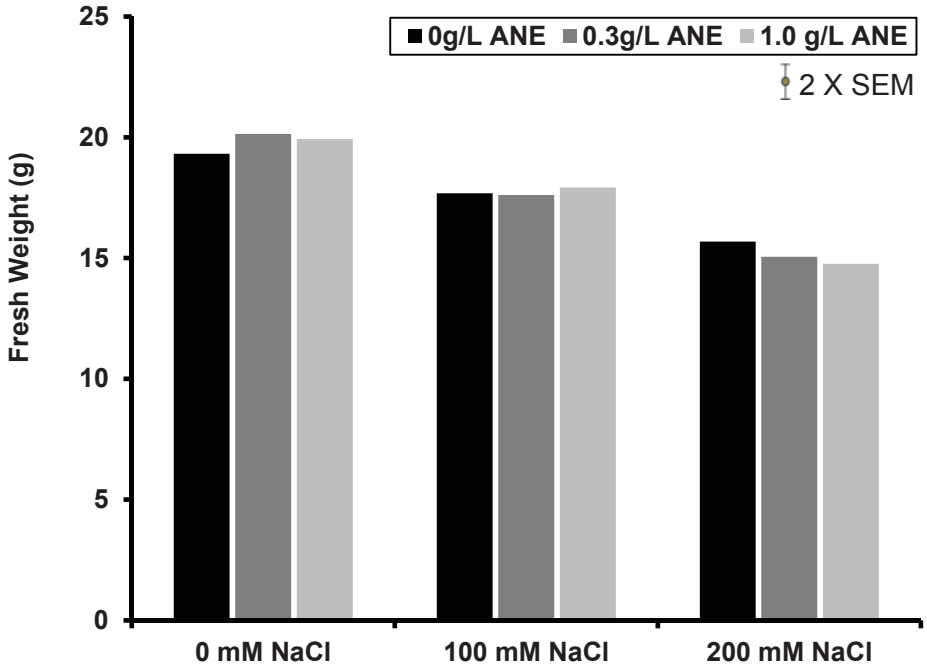


Figure 4.2.1.4: Changes in fresh weight of tomato plants determined after 2 weeks of treatments with NaCl and commercial *Ascophyllum nodosum* extract in greenhouse. Each value represents the average of samples collected from six plants, 2X SEM (±0.433). Treatment combinations of 0 mM, 100 mM and 200 mM of NaCl with 0 g/L, 0.3 g/L and 1.0 g/L were respectively compared.

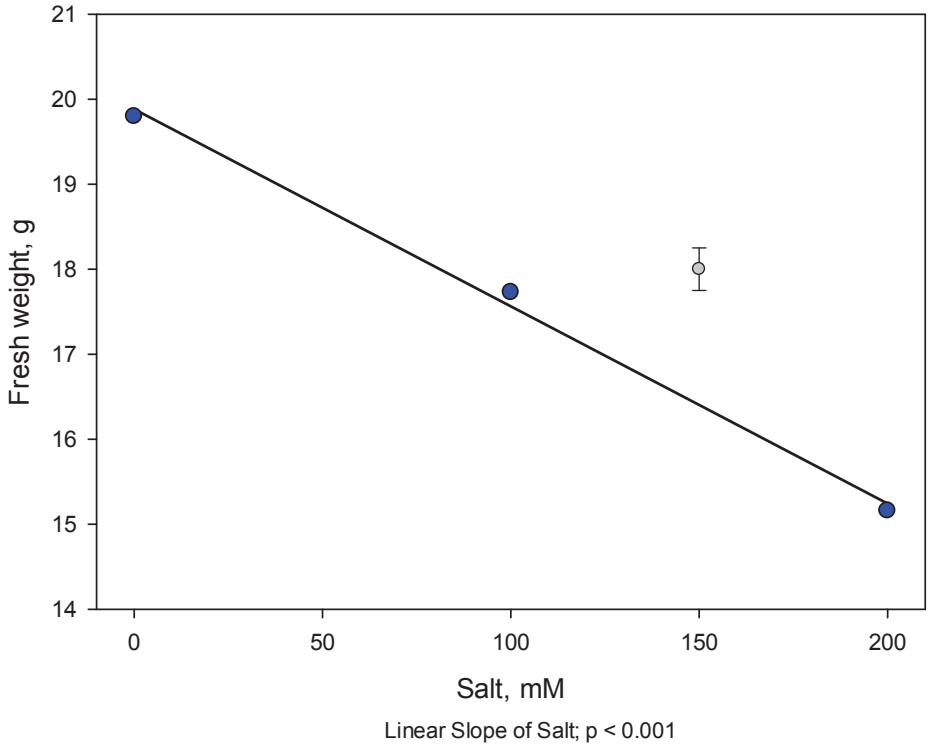


Figure 4.2.1.4a: Linear slope of NaCl concentrations and its effect on fresh weight (g) (p<0.001). Vertical bar represents two standard errors of the mean (± SEM)

4.2.2 Effect of commercial *Ascophyllum nodosum* extract on Na^+ and K^+ content of tomato leaves tissue at 6, 24 and 72 h after treatment

The Na^+ and K^+ content of the leaves were analyzed at 6 h, 24 h and 72 h after application of the treatments. There was no significant difference observed among the six treatments sampled after 6 h. A significant increase in Na^+ level was recorded in 200 mM NaCl treated plants, although no difference was identified between the comparable samples (200 mM NaCl, LANS- 200 mM NaCl and ANE- 200 mM NaCl). All the treatments containing 200 mM NaCl were significantly different at 72 h when compared with 6 h samples. As well, a constant decrease in the concentration of Na^+ was detected in control plants not treated with NaCl, but not with ANE alone, which did not received any NaCl. The test for fixed effects with repeated measures analysis showed significant differences, based on treatment ($p= 0.0001$), time ($p= 0.0696$) and the interaction of time X treatment ($p= 0.0143$).

The amount of K^+ ion absorbed at 6 and 24 h did not show any significant difference between the samples. However, it was significantly higher in the samples containing ANE (ANE alone, ANE-200 mM NaCl) at 72 h. The results for the test for fixed effects were significant for treatment ($p= 0.0001$), time ($p= 0.0001$) and time X treatment interaction ($p= 0.0378$).

It is clear that the NaCl treatments resulted in a higher K^+/Na^+ level at the beginning which decreased as Na^+ content in leaves increased after 72 h. The ANE supplemented NaCl treatments resulted in a higher K^+/ Na^+ ratio at 72 h.

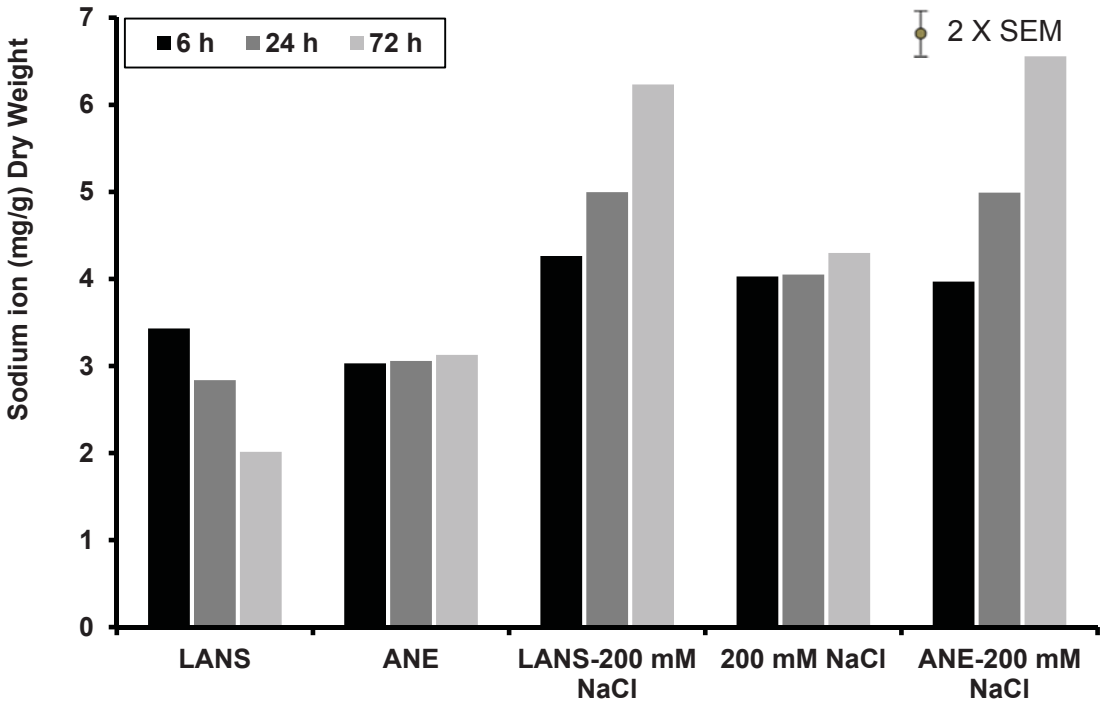


Figure 4.2.2.1: Changes in Na^+ content of the leaves of four week old tomato plants determined at 6 h, 24 h and 72 h following treatments in 200 mM NaCl, with and without ANE supplementation. Each value represents the average of Na^+ content from three replicates and, 2X SEM (±0.208). LANS- Long Ashton Nutrient Solution that contains inorganic ions present in the ANE.

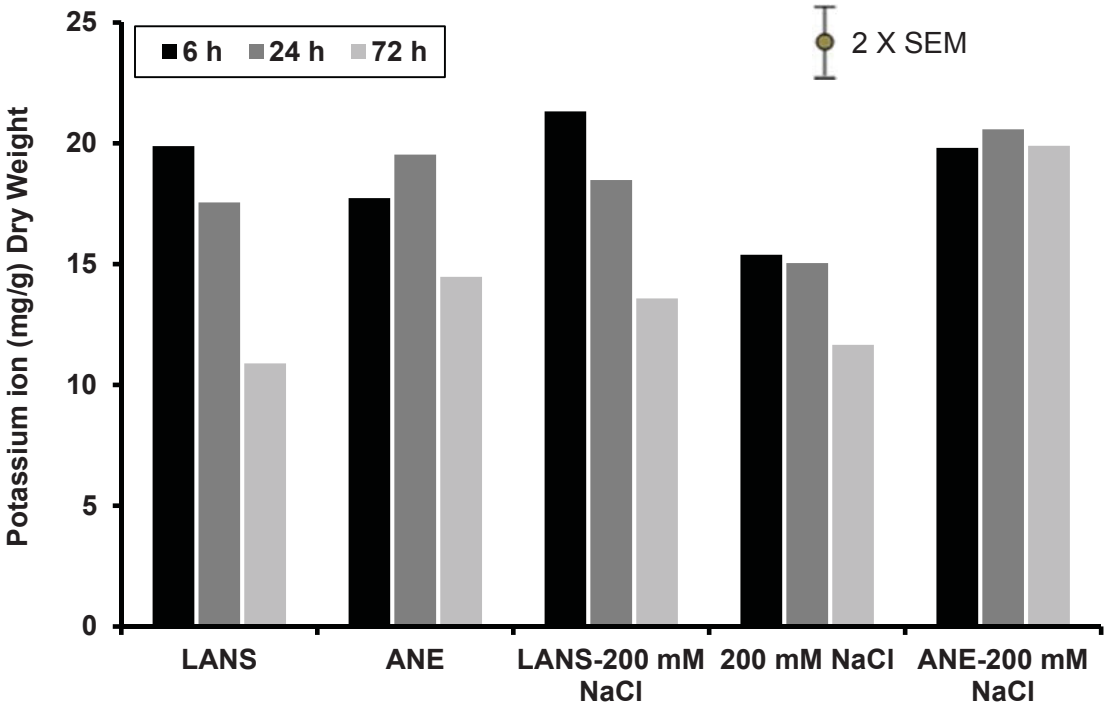


Figure 4.2.2.2: Changes in K^+ content of the leaves of four week old tomato plants determined at 6 h, 24 h and 72 h following treatments in 200 mM NaCl, with and without ANE supplementation. Each value represents the average of Na^+ content from three replicates and, 2X SEM (±1.242). LANS- Long Ashton Nutrient Solution that contains inorganic ions present in the ANE.

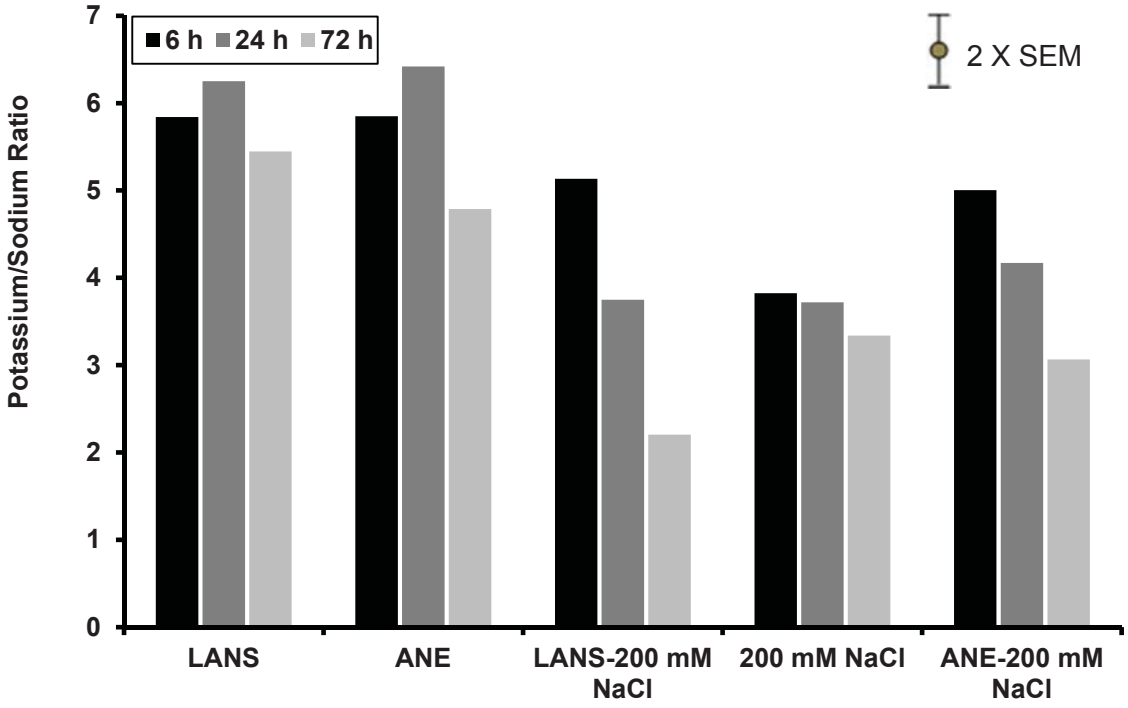


Figure 4.2.2.3: Changes in K^+/Na^+ ratio of the leaves of four week old tomato plants determined at 6 h, 24 h and 72 h following treatments in 200 mM NaCl, with and without ANE supplementation. Each value represents the average of Na^+ content from three replicates and, 2X SEM (±0.468). LANS- Long Ashton Nutrient Solution that contains inorganic ions present in the ANE.

4.3 Effect of EtOAc-ANE and commercial *Ascophyllum nodosum* extract fruit number, yield and biomass of the plants in greenhouse

4.3.1 Effect of EtOAc-ANE on fruit number, yield, fresh weight of plants and total biomass of plant

There was no significant difference in fruit yield **(Figure 4.3.1.1),** number of fruits **(Figure 4.3.1.2)** and plant fresh weight **(Figure 4.3.1.3)** among the treatments

The total aboveground biomass (fruit yield and fresh weight of plants) produced during the study period showed a significant difference (p= 0.0402) between the control and the 200 mM NaCl treatments. No difference was recorded between the control and EtOAc-ANE- 200 mM NaCl group, which might account for the high variance in the data (**Figure 4.3.1.4)**.

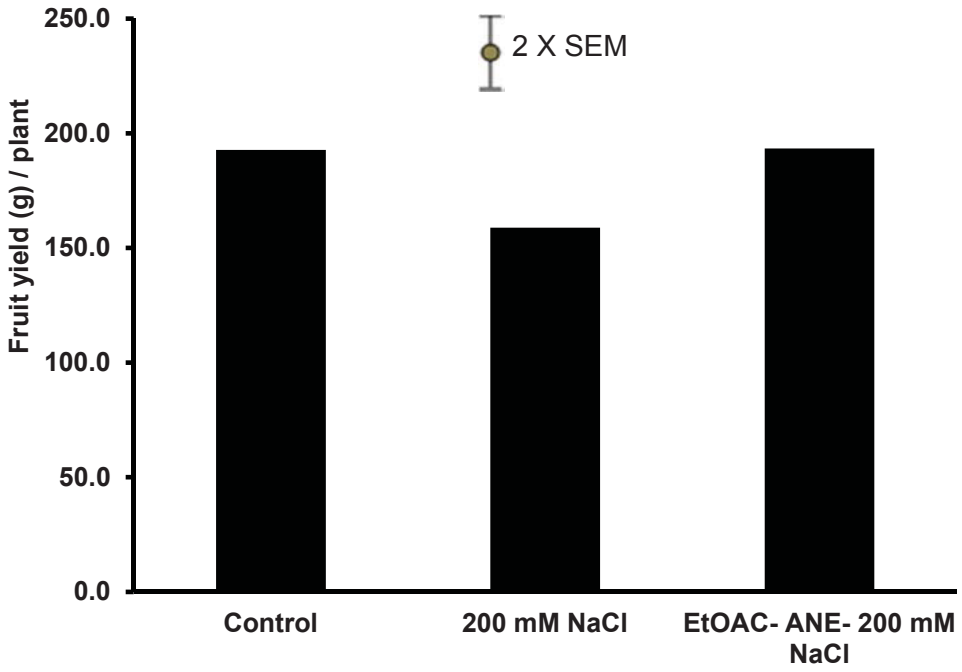


Figure 4.3.1.1 Effect of EtOAc-ANE on fruit yield of plants determined at the end of fruiting in greenhouse condition. Each value represents the average of weights of fruits collected from three replicates, 2X SEM (±17.14). Treatment combinations of 0 mM, 200 mM with and without EtOAC-ANE supplementation were compared.

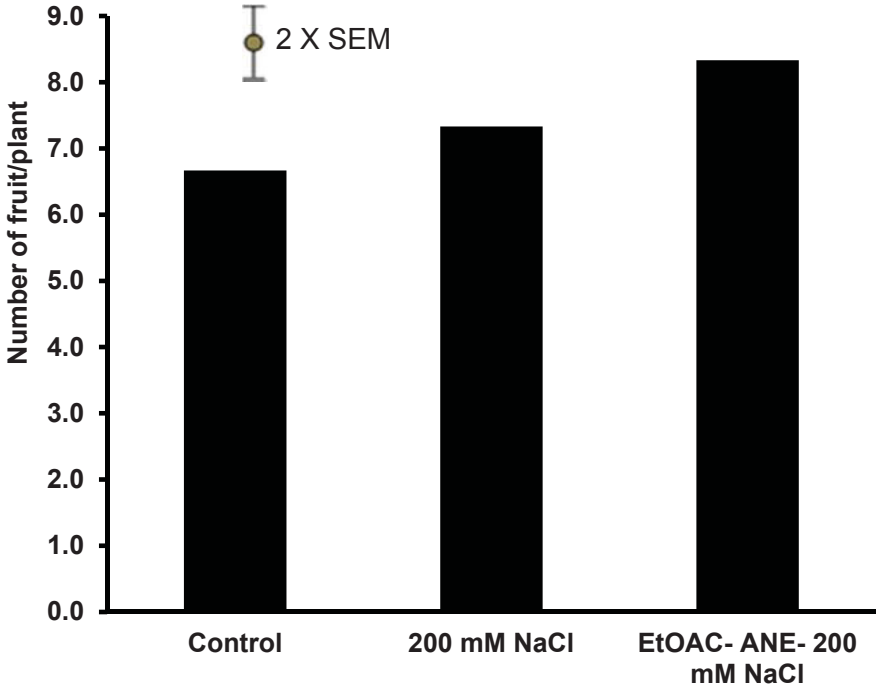


Figure 4.3.1.2 Effect of EtOAc-ANE on number of fruits produced during the study period in greenhouse condition. Each value represents the average of fruits collected from three replicates, 2X SEM (±0.894). Treatment combinations of 0 mM, 200 mM with and without EtOAC-ANE supplementation were compared.

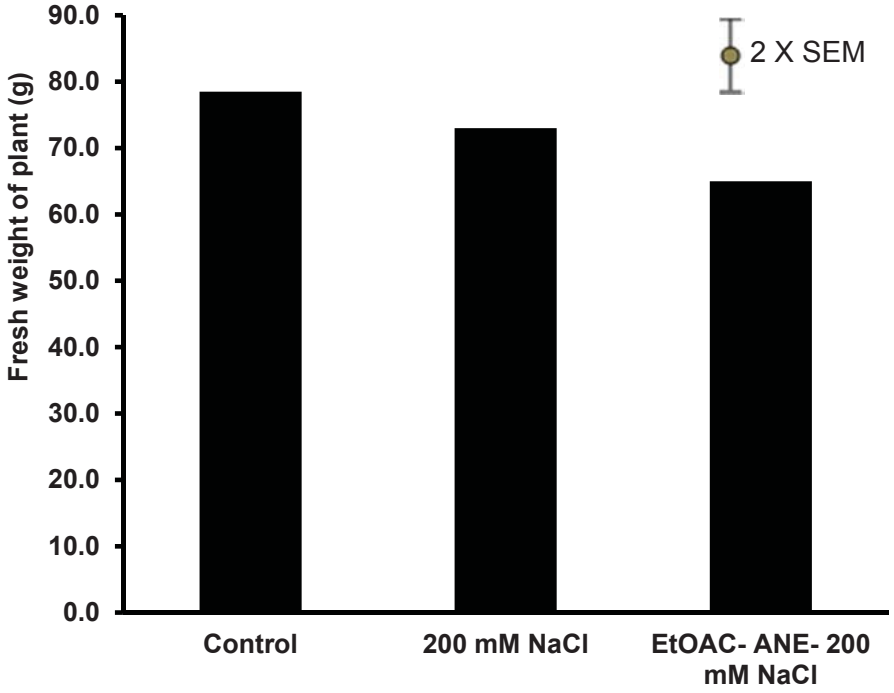


Figure 4.3.1.3 Effect of EtOAc-ANE on fresh weight of plants at the end of the study period. Each value represents the average of plants from three replicates, 2X SEM (±6.585). Treatment combinations of 0 mM, 200 mM with and without EtOAC-ANE supplementation were compared.

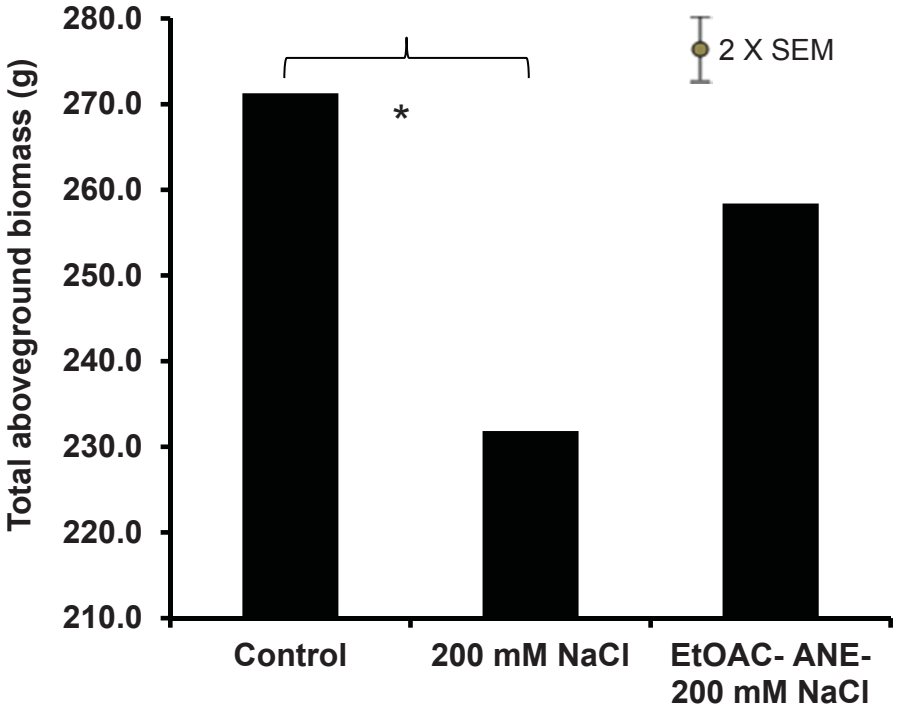


Figure 4.3.1.4 Effect of EtOAc-ANE on total aboveground biomass produced during the study period. Each value represents the average of plants from three replicates, 2X SEM (±14.55). Treatment combinations of 0 mM, 200 mM with and without EtOAC-ANE supplementation were compared. (* = $p < 0.05$).

4.3.2 Effect of commercial *Ascophyllum nodosum* extract on fruit number, yield, fresh weight of plants and total biomass of plant in greenhouse

There was no significant difference recorded in the number of fresh fruits obtained per plant. This data were also close to the number of fruits obtained in greenhouse study using EtOAc- ANE application. LANS treatment was used as an internal inorganic control for ANE.

Interestingly, the mean fresh weight in ANE was similar to that of the EtOAc treatments. A significant difference in the fresh weight of the plants at the end of the experiments was reported. Both salt treated groups (200 mM and 1 g/L ANE-200 mM NaCl) were significantly lower from the controls.

Similarly, no significant difference was observed in the mean fruit yield among the treatments, as seen in the previous parameters.

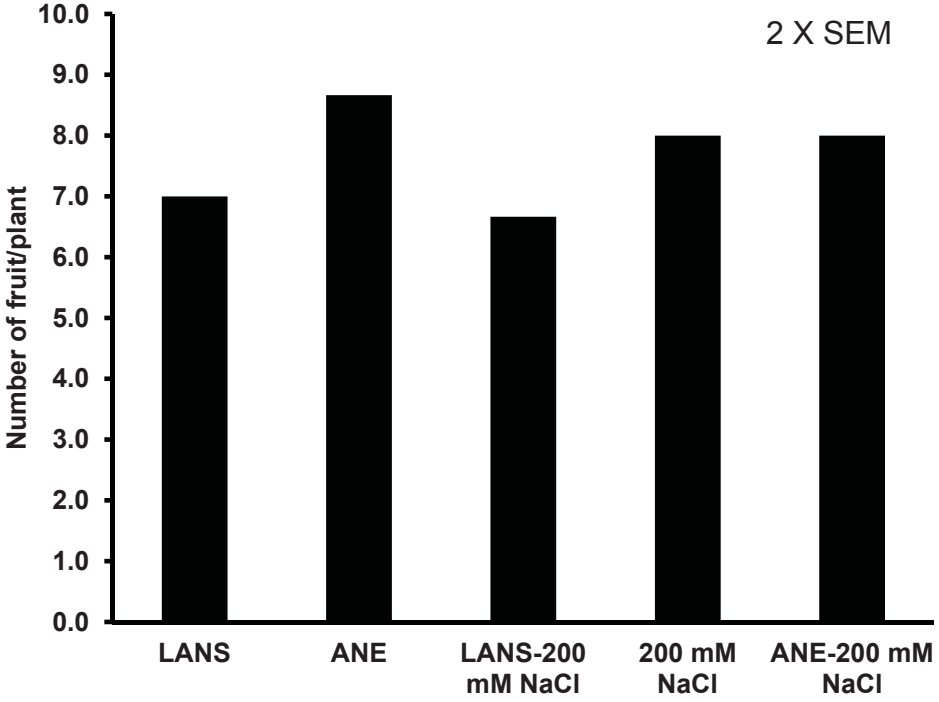


Figure 4.3.2.1: Effect of commercial *Ascophyllum nodosum* extract on number of fruits per plants determined at the end of fruiting in greenhouse condition. Each value represents the average of fruits collected from three replicates, 2X SEM (±1.87). Treatment combinations of 0 mM, 200 mM with and without ANE were compared. ANE- *Ascophyllum nodosum* extract, LANS- Long Ashton Nutrient Solution that contains equivalent inorganic ions present in the ANE.

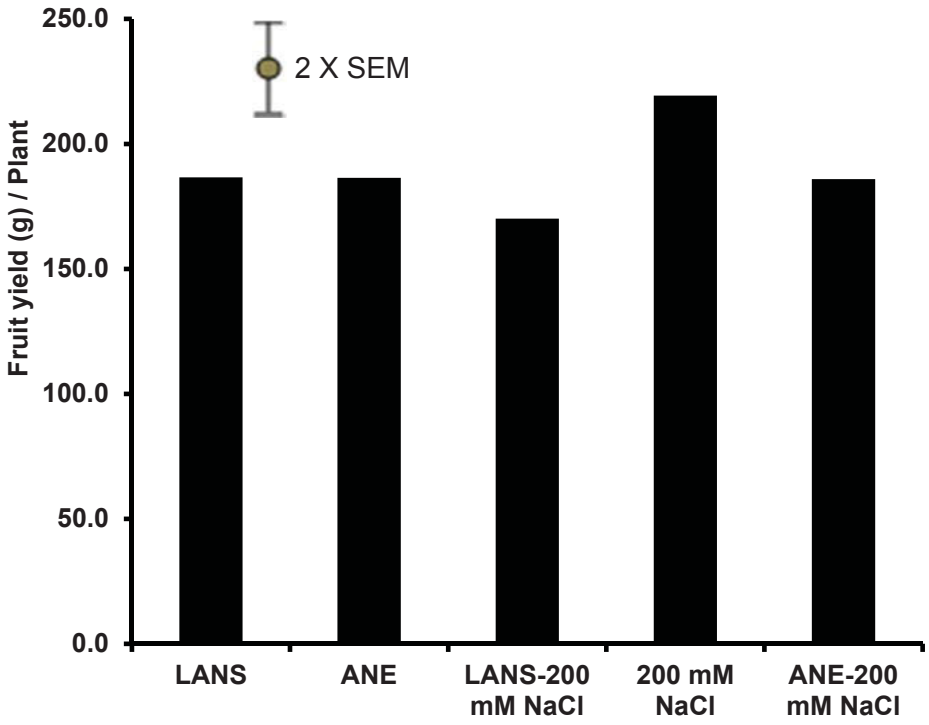


Figure 4.3.2.2: Effect of commercial *Ascophyllum nodosum* extract on fruits yield determined at the end of fruiting period in greenhouse condition. Each value represents the average of fruits collected from three replicates, 2X SEM (±34.76). Treatment combinations of 0 mM, 200 mM with and without ANE were compared. ANE- *Ascophyllum nodosum* extract, LANS- Long Ashton Nutrient Solution that contains equivalent inorganic ions present in the ANE.

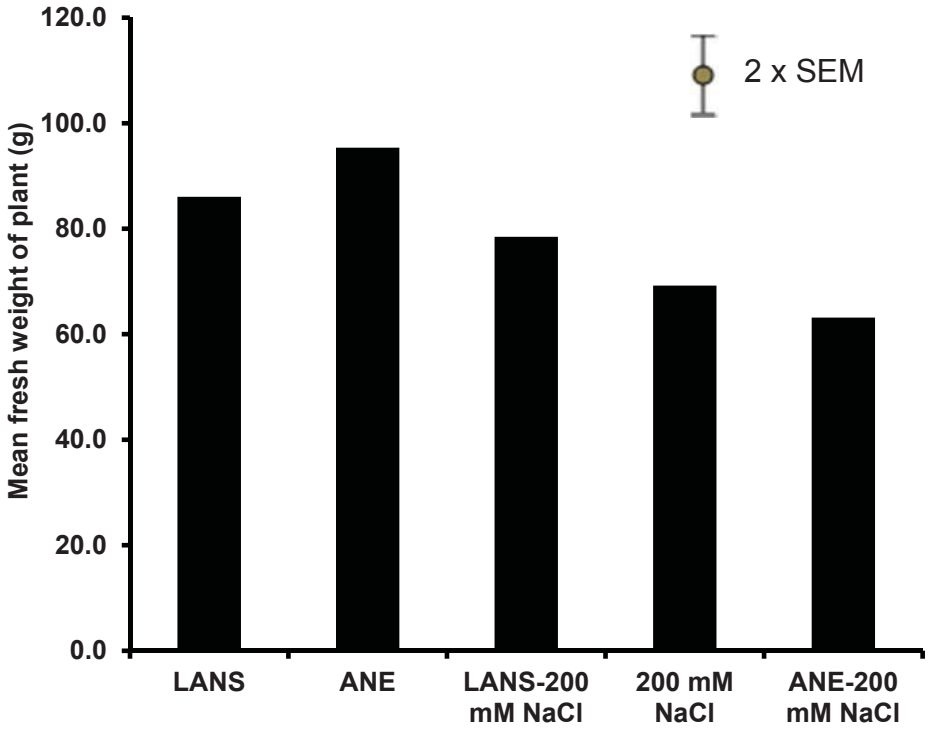


Figure 4.3.2.3 Effect of commercial *Ascophyllum nodosum* extract on fresh weight of plants determined at the end of study. Each value represents the average of three replicates, 2X SEM (±6.80). Treatment combinations of 0 mM, 200 mM with and without ANE were compared. ANE- *Ascophyllum nodosum* extract, LANS- Long Ashton Nutrient Solution that contains equivalent inorganic ions present in the ANE.

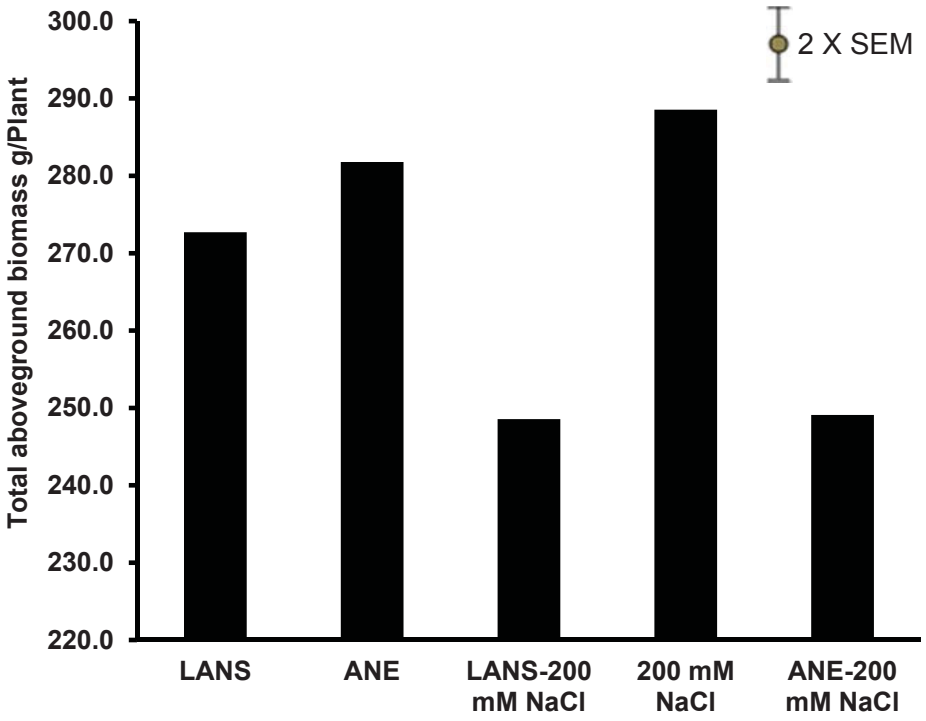


Figure 4.3.2.4: Effect of commercial *Ascophyllum nodosum* extract on total aboveground biomass (fruit yield and fresh weight) determined at the end of study.

Each value represents the average of three replicates, 2X SEM (±31.72). Treatment combinations of 0 mM, 200 mM with and without ANE were compared. ANE- *Ascophyllum nodosum* extract, LANS- Long Ashton Nutrient Solution that contains equivalent inorganic ions present in the ANE.

CHAPTER 5 Discussion

Tomato growth is affected by salts of sodium (NaCl, Na_2CO_3, Na_2SO_4), calcium (Ca_2SO_4), magnesium ($MgSO_4$, $MgCl_2$) and potassium (KCl) (Cuartero et al., 2006; Foolad, 2004; Munns and Tester, 2008; Rengasamy, 2002;). NaCl is the most common salt in saline soils and high concentrations of Na^+ and Cl^- decrease the availability of essential nutrients, such as K^+, Ca^{2+}, Mg^{2+} and NO_3^-. Furthermore, high soil salinity induces osmotic stress in the root zone and reduces the ability of roots to absorb water, leading to a water deficit in the plant. High concentrations of Na^+ and Cl^- affect biochemical processes, inhibit plant growth, and promote early senescence. Extended exposure to high concentrations of Na^+ and Cl^- leads to irreversible cellular damage. Research indicates that sodium ion influx and efflux is related to plants' ability to efficiently compartmentalize and mobilize Na^+ in crop plants such as wheat, rice, barley, beans and tomato (Tester and Davenport, 2003; Apse and Blumwald, 2007).

5.1 EtOAc-ANE improved tomato plant phenotype under salinity stress *in vitro*

Tomato plants, when supplemented with EtOAc-ANE for two weeks, displayed a significant increase in leaf area over the non-supplemented 100 mM NaCl treatments (**Figure 4.1.1.1)**. Leaf area is one of the important criteria for screening plants for tolerance to salinity (Romero-Aranda et al., 2001; Munns, 2002; Cuartero et al., 2006; Munns et al., 2006; Munns and Tester, 2008; Villalta et al., 2008; Albacete et al., 2009). The reduction in leaf area as an effect of high salinity (35 mM – 200 mM) on tomato plants, are similar to other studies on

tomatoes (Li and Stanghellini, 2001; Romero-Aranda et al., 2001; Maggio et al., 2007) and other crop plants such as soybean (Tuncturk et al., 2011), *Phaseolus vulgaris* L. (Seemann and Critchley, 1985), tree species such as olives (Kchaou et al., 2010) and halophytes including *Sesuvium portulacastrum* (Wang et al., 2012).

The reduction in plant growth rate depends on plant species, age, and duration of stress (Munns, 2002). Tomato plants are more susceptible at a younger stage, compared to the flowering and fruiting stages (Dumbroff and Cooper, 1974). Six-week-old tomato plants showed an 8% decrease in total leaf area for every 10 mM NaCl exceeding 65 mM NaCl concentration (Li and Stanghellini, 2001). The present *in vitro* study, with NaCl treatments, has shown an even greater decrease of 12% with every 10 mM increase in salinity levels, which can be explained with the young age (2 weeks) of the plants used in the present *in vitro* study. A number of studies with tomatoes and other crops have used four to six week old plants (Torrecillas et al., 1995; Maiale et al., 2004; Estañ et al., 2005; Juan et al., 2005; Cuartero et al., 2006) and used priming with low levels of salt concentrations (Zapata et al., 2007) before transplanting. Pre-treating plants, or priming seedlings, helps in overcoming the sudden shock injury due to transplants and also imparts tolerance to a number of abiotic stresses (Shannon, 1997; Foolad, 2004; Maurel et al., 2008; Qiu and Yu, 2009; Chen and Polle, 2010; Jones et al., 2012; Nair et al., 2012). The addition of NaCl reduces plant growth rate, which remains lower than that of the unstressed (control) plants (Munns, 2002). It has been observed that plants under salinity stress have

smaller and thicker (succulence) leaves. In the present study, a reduction in plant growth rate was visible after 96 h of salinity imposition, and damage symptoms were clearly visible within a week. Plants were extensively damaged after 14 days of salt exposure which is associated with ion toxicity caused due to accumulation **(Figure 4.1.4)** of ions in the leaves and other parts of the plant (Munns, 2005).

EtOAc-ANE supplementation in 100 mM NaCl treatements significantly increased root length compared to 100 mM NaCl alone **(Figure 4.1.1.2)**. Plant roots is the first site within the plant to be impacted by osmotic changes in the soil environment. Soil water deficit or fluctuations in the ionic profile of the root zone are important factors controlling water movement in the plant root. Thus, monitoring the inhibition of root growth is an important screening criteria for tolerance to salinity (Borsani et al., 2001). Munns, (1993) reported that the extent of root damage is comparatively less than the damage to leaf growth. The present *in vitro* study reported similar findings where the reduction in root length was only 25% **(Figure 4.1.1.2**) compared to the 50% reduction in leaf area when compared to control treatments without NaCl **(Figure 4.1.1.1)**. Several reports which used seaweed extracts have found similar enhancement in the root growth under abiotic stress (Zhang and Schmidt, 1999; Rayorath et al., 2008; Khan et al., 2009; Wally et al., 2012).

5.2 EtOAc-ANE regulates enzymatic activities, metabolites and differential gene expression in tomato plants under salinity stress *in vitro*

Abiotic stress affects many important enzymes in plants such as catalase, peroxidase and superoxide dismutases. These are antioxidative enzymes that

break down H_2O_2 produced in response to drought, salinity and diseases. Therefore, these enzymes are target molecules for determining stress tolerance and their regulation under stress which have been reviewed extensively (Deisseroth and Dounce, 1970; Scandalios et al., 1997; Mittler et al., 1999; Dat et al., 2000; Yang and Poovaiah, 2002; Mittova et al., 2004; Munns and Tester, 2008; Gill and Tuteja, 2010; Rai et al., 2011; Ma et al., 2013). High concentrations of toxic ions result in oxidative stress in cells (Bohnert and Jensen, 1996). The EtOAc-ANE supplemented 100 mM NaCl treatments reported a 33% increase in catalase activity over the control group (100 mM NaCl) at 96 h **(Figure 4.1.2.1**). Interestingly, catalase activity at 96 h was lowered by 34% in control group (100 mM NaCl), when compared to 24 h activity within the same group. This relative decrease in catalase activity in the control plants (100 mM NaCl), compared to the supplemented plants (EtOAc-ANE-100 mM NaCl), suggests that the supplementation of 100 mM NaCl with EtOAc-ANE helped the plants to maintain higher levels of catalase activity in the leaves, protecting from damaging effects of high salt concentration.

This study investigated the transcript abundance of some of the key genes which are differentially regulated by salinity stress imposition in *in vitro* grown tomato plants. The change in transcript abundance of the catalase gene (*catalase-2*) was measured by quantitative real time PCR **(Figure 4.1.5.1)**. A positive correlation of transcript abundance to the enzyme activity was not found and did not correspond to the biochemical activity shown at 24 h or 96 h of treatment **(Figure 4.1.2.1)**. The lower relative quantity can be attributed to the low initial

transcript levels in the samples. Such correlation between the transcript level and protein activity are a center of discussion. The correlation between transcript abundance and protein can be as low as 40% (Hargrove and Schmidt, 1989; Vogel and Marcotte, 2012). Therefore it is possible that the transcript abundance at 96 h post treatment might not correspond to the actual cumulative activity of catalase enzyme **(Figure 4.1.2.1**). Greenbaum et al. (2003), have stated three reasons for such poor correlation in mRNA and protein levels, which includes complicated numerous post transcriptional modifications to form the final functional protein, substantial difference in the *in vivo* half life and finally a siginificantly large error in mapping these two different cellular mechanisms.

Ascophyllum nodosum extracts have been shown to affect the expression of a number of stress responsive genes and activity of anti-stress enzymes (Rayorath et al., 2008; Subramanian et al., 2011; Wally et al., 2012). The activity of Guaiacol peroxidase was reduced in the EtOAc-ANE-100 mM NaCl treatments **(Figure 4.1.2.2)**. Studies have shown similar trends of reduced activity of guaiacol peroxidase, superoxide dismutase and other ROS scavenging enzymes in the root system of tomatoes subjected to high salinity levels (Mittova et al., 2004). ROS damages carbohydrates, DNA, lipids and membrane systems, leading to aberrant signaling. The degree of lipid peroxidation increased equally in both non-treated and EtOAc-ANE supplemented samples. It is possible that the effects of EtOAc-ANE on lipid peroxidation are not observed within the first few days of treatment **(Figure 4.1.2.4)** and another but extended sampling time point such as; seven days will give more insight about the extent of protection

provided by EtOAc-ANE. Interestingly, the rate at which the malondialdehyde (MDA) level increased in control samples was significantly more than NaCl treated plants **(Figure 4.1.2.4)**. EtOAc-ANE supplementation of 100 mM NaCl did not show any change in the MDA content even after 96 h.

Proline, a compatible solute, plays an important role in osmoregulation during water and ionic stress. The effect of ANE-EtOAc on the concentration of proline in tomato leaves was investigated. The stress alleviating effect of proline is well documented in several studies on abiotic stress (Torrecillas et al., 1995; Bohnert and Jensen, 1996; Zhu et al., 1997; Wei et al., 2000; Apse and Blumwald, 2002; Munns, 2002; Ashraf, 2004; Foolad, 2004; Maiale et al., 2004; Mittova et al., 2004; Claussen, 2005; Mahajan and Tuteja, 2005; Parida and Das, 2005; Yamaguchi and Blumwald, 2005; Chen et al., 2009; Khan et al., 2009; Nair et al., 2012). The present *in vitro* study reported 80% increase in the proline content of treatments containing 100 mM NaCl after 96 h **(Figure 4.1.2.3)**. The EtOAc-ANE supplemented 100 mM NaCl treated plants accumulated 15% more proline. This is three-fold less than reported by Rayorath et al. (2008), where studies with freezing tolerance using similar extracts on *Arabidopsis* recorded a 50% increase in proline content after 24 h at -2 ºC. This research has obtained similar results to those reported in the previously described studies. However, the transcript abundance of *tompro-2* gene*,* which codes for Δ^1-pyrroline-5-carboxylate synthetase was low and a correlation could not be established for the bio-chemical abundance at 96 h after treatment.

Salinity stress affects most of the physiological process in plants. Chlorophyll content is one of these important factors. This study estimated the chlorophyll content of salinized plants with similar findings (decrease in treatments without EtOAc-ANE supplementation), as reported by others (Longstreth et al., 1984; Romero-Aranda et al., 2001; Ghanem et al., 2008; Tavakkoli et al., 2010; Tavakkoli et al., 2011). Chlorophyll *a* **(Figure 4.1.3.1)**, chlorophyll *b* **(Figure 4.1.3.2)** and carotenoids were investigated to analyze the underlying mechanisms involved in the rise of chlorophyll levels. Under stress, the degradation of chlorophyll occurs faster than its synthesis. This study reports similar trends in chlorophyll degradation pathways as depicted by Hörtensteiner (2006). An expected increase in chlorophyll *a* concentration at 24 h post 100 mM NaCl treatments **(Figure 4.1.3.1)** was due to chlorophyll *b* degradation **(Figure 4.1.3.2)** (chlorophyll *b* degrades to chlorophyll *a*); conversely chlorophyll *b,* if degraded, was converted to chlorophyll *a.* A significant decrease of chlorophyll *a* after 96 h was observed in plants that did not receive EtOAc-ANE supplementation. Thus, EtOAc-ANE treatments provide significant protection against degradation as no significant changes were observed from 24 h to 96 h after treatment.

The positive effects of *Ascophyllum nodosum* extracts on chlorophyll have been well documented in recent studies (Khan et al., 2009; Khan et al., 2011; Wally et al., 2012; Weeraddana, 2012). A similar chlorophyll *a/b* ratio of (6-7:1) **(Figure 4.1.3.3)** was also observed in this study. This was slightly higher than was reported by (Hendry et al., 1987) which is 4-5:1.

5.3 EtOAc-ANE treatment significantly decreased sodium accumulation in tomato plants under salinity stress *in vitro*

Sodium competes with potassium for binding sites and hampers metabolism by inactivating enzymes and essential cellular functions. Thus, crop growth in salt stress results in injury due to high Na^+ and low K^+ concentrations (Munns and Tester, 2008; Tester and Davenport, 2003). Physiological and biochemical changes occur due to the absorption of toxic levels of sodium, the effects of which have been reviewed extensively (Bernstein, 1975; Shannon, 1997; Shannon and Grieve, 1998; Munns, 2002; Zhu, 2003; Mansour and Salama, 2004; Parida and Das, 2005; Munns and Tester, 2008; Kronzucker and Britto, 2011; Bazihizina et al., 2012; Shahbaz et al., 2012). Cultivated crops are affected to varying degrees by salt stress. This study also reports a decrease in plant growth attributes under salinity stress **(Figure 4.1.1.1; Figure 4.1.1.2)** suggesting that there are negative effects associated with the accumulation of sodium and the time of exposure to salinity. Several studies have reported that genetic transformation (Zhang and Blumwald, 2001; Ouyang et al., 2007; Olias et al., 2009; Belver et al., 2012; Huertas et al., 2012), grafting and rootstock mediation (Estan et al., 2005; Albacete et al., 2009; Asins et al., 2010; Ghanem et al., 2011) can alter the sodium acquisition process, providing tolerance to the plant. A wide range of tomato breeding programs have developed salinity tolerant varieties, focused on pyramiding tolerant traits.

EtoAC-ANE treated plants accumulated significantly less Na^+ 14 days post treatment **(Figure 4.1.4),** as evidenced by the increase in leaf area and root length **(Figure 4.1.1.1; Figure 4.1.1.2)**. The mechanism of entry of ions (mostly,

K^+ and Na^+) into the root space, xylem loading and unloading, overall sodium ion distribution and its compartmentalization in the plant system, have been studied extensively (Niu et al., 1995; Horie and Schroeder, 2004; Cuin and Shabala, 2006; Munns and Tester, 2008; Shabala and Cuin, 2008; Zhang et al., 2009; Craig Plett and Moller, 2010; Hauser and Horie, 2010; Kronzucker and Britto, 2011; Hedrich, 2012). Ions in solution, such as Na^+ and K^+, are hydrated, which prevents easy movement across the hydrophobic lipid bilayer of membranes. Thus, transport proteins are important for ionic fluxes which is guided by the electrical gradient and membrane potential across the membranes. High extracellular Na^+ concentration increases the electrochemical gradient at the membrane and thus, favors passive transport. However, interpreting the effect of EtOAc-ANE is difficult as currently there is little evidence on the direct involvement of a specific class of molecules in the regulation of ion selectivity in plants. Recent experiments with ANE suggest that the chemical components of *Ascophyllum nodosum* extract elicited endogenous biosynthesis of plant hormones (Rayorath et al., 2007; Rayorath et al., 2008; Wally et al., 2012). The presence of NaCl in the growth medium induces ABA in plant systems (Mulholland et al., 2003). Although, hormonal analyses of the plants were not performed, the present research suggests involvement of such plant stress hormones. This present experiment suggests that the induction of stress hormones such as ABA by the application of EtOAc-ANE might have regulated stomatal conductance and thereby, the transpirational pull, which largely contributes to the absorption of water and thus, nutrients and other ions.

5.4 Commercial *Ascophyllum nodosum* extract (ANE) showed complex interaction with NaCl and altered K^+/Na^+ ratio in leaves in the greenhouse

The commercial *Ascophyllum nodosum* extract (ANE) (Acadian, Acadian Seaplants Ltd.) (**Section 3.4**) was used in greenhouse studies on 4 week old tomato plants. Similar and comparable decreases in the leaf area, root length and root surface area were recorded with an increase in salinity from 100 mM to 200 mM NaCl. However, positive effects of ANE on leaf area and root length **(Figure 4.2.1.1; 4.2.1.2; 4.2.1.3)**, in contrast to those seen in EtOAc-ANE supplemented *in vitro* experiments **(Figure 4.1.1.1; 4.1.1.2)**, could not be obtained under greenhouse conditions. Commercial ANE has high ionic (potassium ~ 5%) concentrations which could have increased the threshold levels of sodium absorption, as discussed previously **(Section 5.3)**. A definite linear decrease in plant growth parameters suggested that NaCl had a growth retarding effect in spite of increased applications of ANE. The present greenhouse study detected the interaction of ANE with NaCl and no linear increase in leaf area was recorded, with increasing concentrations of ANE, without NaCl **(Figure 4.2.1.1.)**. The *Ascophyllum nodosum* extracts (ANE) have been used for centuries to alleviate stress periods in plants (Blunden et al., 1985; Hurtado et al., 2009; Subramanian, 2008; Khan et al., 2009; Rayirath, 2009; Craigie, 2011; Fan, 2010; Subramanian et al., 2011; Nair et al., 2012; Wally et al., 2012). The presence of a diverse group of organic compounds in seaweed extracts, and their interactions, have been correlated to such stress tolerance

(Blunden et al., 1985; Craigie et al., 2008; MacKinnon et al., 2009; Craigie, 2011).

The leaf tissues were sampled at different time intervals (6 h, 24 h, and 72 h) from the plants grown in the greenhouse. The samples were analyzed for presence of Na^+ and K^+. The results obtained in this greenhouse experiment are unique as there was no decrease in the concentration of Na^+ in the leaves of plants from 6 h – 72 h, when only ANE was applied as treatment **(Figure 4.2.2.1)**. It is suggested that the leaf expansion of these plants containing ANE, was halted, or slowed significantly **(Figure 4.2.2.1)**. This observation was similar to the treatment group containing 200 mM NaCl. In plants containing LANS as a treatment, a consistent decrease in the Na^+ content was recorded because of the plants' continuous growth, which diluted the ion concentration per gram dry weight. The supplementation of plants with ANE and LANS, with 200 mM NaCl, made it difficult for plants to respond to ionic changes, as both treatments (ANE and LANS) contained higher potassium (~5%), due to the alkaline nature of the commercial product used. Thus, the high potassium **(Figure 4.2.2.2)** in the treatments containing ANE or LANS might have increased the threshold levels of Na^+ intake, and disturbed the stress signaling pathways, thereby, damaging plants in the long run. This is because, the potassium ion is easily translocated to different growing parts of the plant, leading to an unbalanced cellular ionic state. This would eventually lead to cellular damage. Interestingly, the K^+ content remained at the same level for treatment containing ANE-200 mM NaCl **(Figure 4.2.2.2)**, which points that application of ANE contributed to maintainance of high

K^+ levels for at least 72 h post treatment. Under normal plant growth conditions, a high cytosolic K^+/Na^+ was maintained, which varied from (100-200 mM K^+) / (1-10 mM Na^+). This study reported lower K^+/Na^+ ratio, the maximum of which was ~6 observed in ANE treatments and ~2.5 in LANS treatments, emphasizing that the external concentration of NaCl used in the study was very high, with respect to normal growing conditions.

5.5 Effects of ethyl acetate organic fraction (EtOAc-ANE) and *Ascophyllum nodosum* extracts (ANE) on tomato yield

Yield is an important factor in choosing a crop variety for cultivation. Most of the research related to salinity stress aims to improve crop yield. Several researchers have reported a decrease in yield, even at low sodium ion concentrations (Cuartero and Fernández-Muñoz, 1998; Balibrea et al., 2000; Li and Stanghellini, 2001; Zhang and Blumwald, 2001; Estan et al., 2005; Albacete et al., 2009; Estan et al., 2009; Lu et al., 2010; Ghanem et al., 2011; Lovelli et al., 2012). The yield studies were conducted in the summer months when the greenhouses had high temperatures (30-35 °C). Interestingly, no differences were observed in the salinized plants, which performed at par with the control plants **(Section 4.3.1; 4.3.2)**. A recent study (Rivero et al., 2013) revealed a specific physiological, biochemical and molecular response in tomato plants when triggered by the combined effects of salinity and heat. This experiment could be summarized as the outcome of the sum of all different factors leading to salinity tolerance. Rivero et al. (2013) reported that the accumulation of betaine and trehalose was directly correlated to the maintenance of a high potassium to sodium ion ratio. The present greenhouse experiment has empirical similarities to

long periods of high temperature stress, coupled with high salinity stress as shown by Rivero et al. (2013).

CHAPTER 6 Conclusion and Summary

Soil salinity is one of the widely faced challenges in present agriculture. The reduced productivity of crops under saline growth conditions are functions of growth attributes such as germination, growth rate, photosynthetic efficiency and biomass accumulation and are extensively researched in most abiotic stress related crop studies. The application of seaweed and seaweed products has been documented to alleviate a wide range of abiotic stresses. *Ascophyllum nodosum* is used in agriculture as plant biostimulant (Craige, 2011). *A. nodosum* extract (ANE), stimulates shoot growth and branching, and improves nutrient uptake.

The findings of the present study confirmed that the supplementation of ethyl acetate subfractions of the *Ascophyllum nodosum* extract significantly increased leaf area and root length of two week old plants, grown in 100 mM NaCl, in *in vitro* conditions. The application of EtOAc-ANE also reduced *in-planta* concentration of Na^+ in the plants. Further research will be required to establish the molecular basis of such ion selectivity. The seedlings showed higher catalase activity than the non supplemented 100 mM NaCl control plants. Moreover, the supplemented samples retained significantly higher chlorophyll (chl*a*, chl*b* and carotenoids) than non-supplemented stressed plants after 96 h of exposure to treatments. An increase of 15% in proline level was also recorded 100 mM NaCl-EtOAc-ANE treated plants compared to the 100 mM NaCl control.

The greenhouse studies with the commercial *Ascophyllum nodosum* extract (ANE) (Acadian™, Acadian Seaplants Limited, Nova Scotia, Canada) showed

interaction with the salt concentartions used in the study (100 mM and 200 mM NaCl). The increasing NaCl concentration, irrespective of the concentartion of ANE used, showed significant linear decrease in the plant phenotypic characters (leaf area, root length, root area, fresh weight) studied. Also, no increase in the such charactersistics were recorded when only ANE was applied which confirms that the ionic composition of ANE were not sufficient to produce any phentotypic changes but significantly affected the ionic balance of Na^+ and K^+. The ANE treated plants were able to maintain higher K^+ content at all the sampling time points. The older leaves showed faster yellowing (loss of chlorophyll) than non ANE treated leaves possibly as an after effect of accumulation of higher Na^+ content.

The greenhouse studies conducted to study the affect of EtOAc-ANE and Acadian™ on fruit number, yield and plant biomass, did not show any significant diffecrence. The experiment was conducted in the summer months when the greenhouses had high temperatures (30-35 °C). This greenhouse study was similar to a recent research (Rivero et al., 2013) which revealed tomato plants when triggered by the combined effects of salinity and heat, were able to perform as par as control plants.

REFERENCES

Ahmad, P., Jaleel, C. A., Salem, M. A., Nabi, G., Sharma, S. 2010. Roles of enzymatic and nonenzymatic antioxidants in plants during abiotic stress. Crit. Rev. Biotechnol. **30**(3): 161-175.

Ahmad, P., Sarwat, M., Sharma, S. 2008. Reactive oxygen species, antioxidants and signaling in plants. Journal of Plant Biology **51**(3): 167-173.

Albacete, A., Martínez-Andújar, C., Ghanem, M. E., Acosta, M., Sánchez-Bravo, J., Asins, M. J., Cuartero, J., Lutts, S., Dodd, I. C., Pérez-Alfocea, F. 2009. Rootstock-mediated changes in xylem ionic and hormonal status are correlated with delayed leaf senescence, and increased leaf area and crop productivity in salinized tomato. Plant, Cell Environ. **32**(7): 928-938.

Amtmann, A. and Sanders, D. 1998. Mechanisms of Na^+ uptake by plant cells. Adv. Bot. Res. **29**: 75-112.

Apse, M. P. and Blumwald, E. 2002. Engineering salt tolerance in plants. Curr. Opin. Biotechnol. **13**(2): 146-150.

Apse, M. P., Aharon, G. S., Snedden, W. A., Blumwald, E. 1999. Salt tolerance conferred by over-expression of a vacuolar Na /H antiport in *Arabidopsis*. Science **285**(5431): 1256-1258.

Asada, K. 1992. Ascorbate peroxidase–a hydrogen peroxide-scavenging enzyme in plants. Physiol. Plantarum **85**(2): 235-241.

Ashraf, M. 2009. Biotechnological approach of improving plant salt tolerance using antioxidants as markers. Biotechnol. Adv. **27**(1): 84-93.

Ashraf, M. 2004. Some important physiological selection criteria for salt tolerance in plants. Flora-Morphology, Distribution, Functional Ecology of Plants **199**(5): 361-376.

Asins, M., Bolarín, M., Pérez-Alfocea, F., Estan, M., Martínez-Andújar, C., Albacete, A., Villalta, I., Bernet, G., Dodd, I. C., Carbonell, E. 2010. Genetic analysis of physiological components of salt tolerance conferred by solanum rootstocks. What is the rootstock doing for the scion? Theor. Appl. Genet. **121**(1): 105-115.

Aubert, S., Hennion, F., Bouchereau, A., Gout, E., Bligny, R., Dorne, A. 1999. Subcellular compartmentation of proline in the leaves of the subantarctic kerguelen cabbage *Pringlea antiscorbutica* R. br. in vivo 13C ‑ NMR study. Plant, Cell Environ. **22**(3): 255-259.

Ayers, A. D., Brown, J., Wadleigh, C. 1952. Salt tolerance of barley and wheat in soil plots receiving several salinization regimes. Agron. J. **44**(6): 307-310.

Balibrea, M. E., Dell'Amico, J., Bolarín, M. C., Pérez-Alfocea, F. 2000. Carbon partitioning and sucrose metabolism in tomato plants growing under salinity. Physiol. Plantarum **110**(4): 503-511.

Bazihizina, N., Barrett-Lennard, E. G., Colmer, T. D. 2012. Plant growth and physiology under heterogeneous salinity. Plant Soil **354**(1-2): 1-19.

Belver, A., Olías, R., Huertas, R., Rodríguez-Rosales, M. P. 2012. Involvement of *SlSOS2* in tomato salt tolerance. Bioengineered **3**(5): 298-302.

Bernstein, L. 1975. Effects of salinity and sodicity on plant growth. Annu. Rev. Phytopathol. **13**(1): 295-312.

Blumwald, E., Aharon, G. S., Apse, M. P. 2000. Sodium transport in plant cells. Biochimica Et Biophysica Acta (BBA)-Biomembranes **1465**(1): 140-151.

Blunden, G., Gordon, S. M., Smith, B. E., Fletcher, R. L. 1985. Quaternary ammonium compounds in species of the fucaceae (phaeophyceae) from Britain. British Phycological Journal **20**(2): 105-108.

Bohnert, H. J. and Jensen, R. G. 1996. Strategies for engineering water-stress tolerance in plants. Trends Biotechnol. **14**(3): 89-97.

Borsani, O., Cuartero, J., Fernández, J. A., Valpuesta, V., Botella, M. A. 2001. Identification of two loci in tomato reveals distinct mechanisms for salt tolerance. The Plant Cell Online **13**(4): 873-887.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**(1): 248-254.

Brady, N. C. and Weil, R. R. 2010. Elements of the nature and properties of soils. Pearson Educational International. Upper Saddle River, NJ

Bruns, S. and Hecht-Buchholz, C. 1990. Light and electron microscope studies on the leaves of several potato cultivars after application of salt at various development stages. Potato Res. **33**(1): 33-41.

Bui, E. 2013. Soil salinity: A neglected factor in plant ecology and biogeography. J. Arid Environ. **92**: 14-25.

Cantore, V., Boari, F. and Pace, B. 2005. Salinity effects on tomato. XV meeting of the EUCARPIA tomato working group 789.

Chen, G. and Asada, K. 1989. Ascorbate peroxidase in tea leaves: Occurrence of two isozymes and the differences in their enzymatic and molecular properties. Plant and Cell Physiology **30**(7): 987-998.

Chen, S. and Polle, A. 2010. Salinity tolerance of populus. Plant Biology **12**(2): 317-333.

Chen, S., Gollop, N., Heuer, B. 2009. Proteomic analysis of salt-stressed tomato (*Solanum lycopersicum*) seedlings: Effect of genotype and exogenous application of glycinebetaine. J. Exp. Bot. **60**(7): 2005-2019.

Chomczynski, P. and Sacchi, N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**(1): 156-159.

Christmann, A., Weiler, E. W., Steudle, E., Grill, E. 2007. A hydraulic signal in root ‐ to ‐ shoot signaling of water shortage. The Plant Journal **52**(1): 167-174.

Claussen, W. 2005. Proline as a measure of stress in tomato plants. Plant Science **168**(1): 241-248.

Craig Plett, D. and Møller, I. S. 2010. Na transport in glycophytic plants: What we know and would like to know. Plant, Cell Environ. **33**(4): 612-626.

Craigie, J. S. 2011. Seaweed extract stimuli in plant science and agriculture. J. Appl. Phycol. **23**(3): 371-393.

Craigie, J. S., MacKinnon, S. L., Walter, J. A. 2008. Liquid seaweed extracts identified using 1H NMR profiles. J. Appl. Phycol. **20**(5): 665-671.

Cram, W. 1983. Chloride accumulation as a homeostatic system: Set points and perturbations the physiological significance of influx isotherms, temperature effects and the influence of plant growth substances. J. Exp. Bot. **34**(11): 1484-1502.

Crouch, I., Beckett, R., Van Staden, J. 1990. Effect of seaweed concentrate on the growth and mineral nutrition of nutrient-stressed lettuce. J. Appl. Phycol. **2**(3): 269-272.

Cuartero, J. and Fernández-Muñoz, R. 1998. Tomato and salinity. Scientia Horticulturae **78**(1): 83-125.

Cuartero, J., Bolarin, M., Asins, M., Moreno, V. 2006. Increasing salt tolerance in the tomato. J. Exp. Bot. **57**(5): 1045-1058.

Cuin, T. A. and Shabala, S. 2006. Potassium homeostasis in salinized plant tissues. Pages 287-317 *in:* Plant electrophysiology. Springer Berlin Heidelberg.

Dat, J., Vandenabeele, S., Vranova, E., Van Montagu, M., Inzé, D., Van Breusegem, F. 2000. Dual action of the active oxygen species during plant stress responses. Cellular and Molecular Life Sciences CMLS **57**(5): 779-795.

De Bruxelles, G., Peacock, W. J., Dennis, E. S., Dolferus, R. 1996. Abscisic acid induces the alcohol dehydrogenase gene in *Arabidopsis*. Plant Physiol. **111**(2): 381-391.

Deckert, R. and Garbary, D. 2005. *Ascophyllum* and its symbionts. VI. Microscopic characterization of the *Ascophyllum nodosum* (phaeophyceae), *Mycophycias ascophylli* (ascomycetes) symbiotum. Algae-Inchon- **20**(3): 225.

Deisseroth, A. and Dounce, A. 1970. Catalase: Physical and chemical properties, mechanism of catalysis, and physiological role. Physiol. Rev. **50**(3): 319-375.

Dietz, K., Tavakoli, N., Kluge, C., Mimura, T., Sharma, S., Harris, G., Chardonnens, A., Golldack, D. 2001. Significance of the V-type ATPase for the adaptation to stressful growth conditions and its regulation on the molecular and biochemical level. J. Exp. Bot. **52**(363): 1969-1980.

Dumbroff, E. and Cooper, A. 1974. Effects of salt stress applied in balanced nutrient solutions at several stages during growth of tomato. Botanical Gazette: 219-224.

Epstein, E. 1966. Dual pattern of ion absorption by plant cells and by plants. .

Estañ, M. T., Martinez-Rodriguez, M. M., Perez-Alfocea, F., Flowers, T. J., Bolarin, M. C. 2005. Grafting raises the salt tolerance of tomato through limiting the transport of sodium and chloride to the shoot. J. Exp. Bot. **56**(412): 703-712.

Estañ, M., Villalta, I., Bolarín, M., Carbonell, E., Asins, M. 2009. Identification of fruit yield loci controlling the salt tolerance conferred by solanum rootstocks. Theor. Appl. Genet. **118**(2): 305-312.

Fan, D. 2010. *Ascophyllum nodosum* extracts improve shelf life and nutritional quality of spinach (spinacia oleracea L.). http://dalspace.library.dal.ca/handle/10222/13120

Fike, J., Allen, V., Schmidt, R., Zhang, X., Fontenot, J., Bagley, C., Ivy, R., Evans, R., Coelho, R., Wester, D. 2001. Tasco-forage: I. influence of a seaweed extract on antioxidant activity in tall fescue and in ruminants. J. Anim. Sci. **79**(4): 1011-1021.

Flowers, T. and Yeo, A. 1995. Breeding for salinity resistance in crop plants: Where next? Functional Plant Biology **22**(6): 875-884.

Flowers, T. and Dalmond, D. 1992. Protein synthesis in halophytes: The influence of potassium, sodium and magnesium in vitro. Plant Soil **146**(1-2): 153-161.

Flowers, T. J. 2004. Improving crop salt tolerance. J. Exp. Bot. **55**(396): 307-319.

Foolad, M. 2004. Recent advances in genetics of salt tolerance in tomato. Plant Cell, Tissue and Organ Culture **76**(2): 101-119.

Ford, C. W. 1984. Accumulation of low molecular weight solutes in water-stressed tropical legumes. Phytochemistry **23**(5): 1007-1015.

Fricke, W., Akhiyarova, G., Veselov, D., Kudoyarova, G. 2004. Rapid and tissue-specific changes in ABA and in growth rate in response to salinity in barley leaves. J. Exp. Bot. **55**(399): 1115-1123.

Fricke, W., Akhiyarova, G., Wei, W., Alexandersson, E., Miller, A., Kjellbom, P. O., Richardson, A., Wojciechowski, T., Schreiber, L., Veselov, D. 2006. The short-term growth response to salt of the developing barley leaf. J. Exp. Bot. **57**(5): 1079-1095.

Fujita, T., Maggio, A., Garcia-Rios, M., Bressan, R. A., Csonka, L. N. 1998. Environmental and stress physiology-comparative analysis of the regulation of expression and structures of two evolutionarily divergent genes for D1-pyrroline-5-carboxylate synthetase from tomato. Plant Physiol. **118**(2): 661-674.

Garbary, D. and Deckert, R. 2004. Three part harmony—*Ascophyllum* and its symbionts. Pages 309-321 *in:* Symbiosis. Springer Netherlands.

Garciadeblas, B., Senn, M. E., Banuelos, M. A., Rodríguez - Navarro, A. 2003. Sodium transport and HKT transporters: The rice model. The Plant Journal **34**(6): 788-801.

Garbary, D. and Gautam, A. 1989. The *Ascophyllum*, *polysiphonia*, *mycosphaerella* symbiosis. I. population ecology of *Mycosphaerella* from Nova Scotia. Bot. Mar. **32**(2): 181-186.

Garg, A. K., Kim, J. K., Owens, T. G., Ranwala, A. P., Choi, Y. D., Kochian, L. V., Wu, R. J. 2002. Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. Proc. Natl. Acad. Sci. U. S. A. **99**(25): 15898-15903.

Ghanem, M. E., Albacete, A., Martínez-Andújar, C., Acosta, M., Romero-Aranda, R., Dodd, I. C., Lutts, S., Pérez-Alfocea, F. 2008. Hormonal changes during salinity-induced leaf senescence in tomato (*Solanum lycopersicum* L.). J. Exp. Bot. **59**(11): 3039-3050.

Ghanem, M. E., Albacete, A., Smigocki, A. C., Frébort, I., Pospíšilová, H., Martínez-Andújar, C., Acosta, M., Sánchez-Bravo, J., Lutts, S., Dodd, I. C. 2011. Root-synthesized cytokinins improve shoot growth and fruit yield in salinized tomato (*Solanum lycopersicum* L.) plants. J. Exp. Bot. **62**(1): 125-140.

Ghassemi, F., Jakeman, A.J., and Nix, H.A. 1995. Stalinization of Land and Water Resources. University of New South Wales Press Ltd, Canberra, Australia.

Gill, S. S. and Tuteja, N. 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiology and Biochemistry **48**(12): 909-930.

Ginzberg, I., Stein, H., Kapulnik, Y., Szabados, L., Strizhov, N., Schell, J., Koncz, C., Zilberstein, A. 1998. Isolation and characterization of two different cDNAs of Δ1-pyrroline-5-carboxylate synthase in Alfalfa, transcriptionally induced upon salt stress. Plant Mol. Biol. **38**(5): 755-764.

Glenn, E. P., Brown, J. J., Blumwald, E. 1999. Salt tolerance and crop potential of halophytes. Crit. Rev. Plant Sci. **18**(2): 227-255.

Gómez‐Cadenas, A., Tadeo, F., Primo‐Millo, E., Talon, M. 1998. Involvement of abscisic acid and ethylene in the responses of citrus seedlings to salt shock. Physiol. Plantarum **103**(4): 475-484.

Gómez-Cadenas, A., Arbona, V., Jacas, J., Primo-Millo, E., Talon, M. 2002. Abscisic acid reduces leaf abscission and increases salt tolerance in citrus plants. J. Plant Growth Regul. **21**(3): 234-240.

Greenbaum, D., Colangelo, C., Williams, K., Gerstein, M. 2003. Comparing protein abundance and mRNA expression levels on a genomic scale. Genome Biol. **4**(9): 117.

Greenway, H. and Munns, R. 1980. Mechanisms of salt tolerance in nonhalophytes. Annual Review of Plant Physiology **31**(1): 149-190.

Haberern, J. 1992. A soil health index. J. Soil Water Conserv. **47**(1): 6.

Halliwell, B. and Gutteridge, J. 1985. Free Radicals in Biology and Medicine. Clarendon Press (Oxford and New York).

Hargrove, J. L. and Schmidt, F. H. 1989. The role of mRNA and protein stability in gene expression. The FASEB Journal **3**(12): 2360-2370.

Hasegawa, M., Bressan, R., Pardo, J. M. 2000. The dawn of plant salt tolerance genetics. Trends Plant Sci. **5**(8): 317-319.

Hauser, F. and Horie, T. 2010. A conserved primary salt tolerance mechanism mediated by HKT transporters: A mechanism for sodium exclusion and maintenance of high K /Na ratio in leaves during salinity stress. Plant, Cell Environ. **33**(4): 552-565.

Hawkins, H. and Lewis, O. 1993. Combination effect of NaCl salinity, nitrogen form and calcium concentration on the growth, ionic content and gaseous exchange properties of *Triticum aestivum* L. cv. gamtoos. New Phytol. **124**(1): 161-170.

Hayashi, H., Mustardy, L., Deshnium, P., Ida, M., Murata, N. 1997. Transformation of *Arabidopsis thaliana* with the *codA* gene for choline oxidase; accumulation of glycinebetaine and enhanced tolerance to salt and cold stress. The Plant Journal **12**(1): 133-142.

Hedrich, R. 2012. Ion channels in plants. Physiol. Rev. **92**(4): 1777-1811.

Hernandez, J., Campillo, A., Jimenez, A., Alarcon, J., Sevilla, F. 1999. Response of antioxidant systems and leaf water relations to NaCl stress in pea plants. New Phytol. **141**(2): 241-251.

Hernandez, J., Campillo, A., Jimenez, A., Alarcon, J., Sevilla, F. 1999. Response of antioxidant systems and leaf water relations to NaCl stress in pea plants. New Phytol. **141**(2): 241-251.

Hendry, G. A., Houghton, J. D., Brown, S. B. 1987. Tansley review no. 11. The degradation of chlorophyll-*a* biological enigma. New Phytol. : 255-302.

Hiscox, J. and Israelstam, G. 1979. A method for the extraction of chlorophyll from leaf tissue without maceration. Canadian Journal of Botany **57**(12): 1332-1334.

Horie, T. and Schroeder, J. I. 2004. Sodium transporters in plants. Diverse genes and physiological functions. Plant Physiol. **136**(1): 2457-2462.

Hörtensteiner, S. 2006. Chlorophyll degradation during senescence. Annu.Rev.Plant Biol. **57**: 55-77.

Huertas, R., Olias, R., Eljakaoui, Z., Gálvez, F. J., Li, J., De Morales, P. A., Belver, A., Rodríguez-Rosales, M. P. 2012. Overexpression of SlSOS2 (SlCIPK24) confers salt tolerance to transgenic tomato. Plant, Cell Environ. **35**(8): 1467-1482.

Hurtado, A. Q., Yunque, D. A., Tibubos, K., Critchley, A. T. 2009. Use of Acadian marine plant extract powder from *Ascophyllum nodosum* in tissue culture of kappaphycus varieties. J. Appl. Phycol. **21**(6): 633-639.

Iyengar, E. and Reddy, M. 1996. Photosynthesis in highly salt tolerant plants. Handbook of Photosynthesis. Marshal Dekar, Baten Rose, USA **909**.

John, J. A and Williams, E. R. 1995. Cyclic and Computer Generated Designs, 2nd Edition, Chapman & Hall, 2-6 Boundary Row, London SE1 8HN, UK

Johnson, M. K., Johnson, E. J., MacElroy, R. D., Speer, H. L., Bruff, B. S. 1968. Effects of salts on the halophilic alga dunaliella viridis. J. Bacteriol. **95**(4): 1461-1468.

Jones, D. L., Hodge, A., Kuzyakov, Y. 2004. Plant and mycorrhizal regulation of rhizodeposition. New Phytol. **163**(3): 459-480.

Juan, M., Rivero, R. M., Romero, L., Ruiz, J. M. 2005. Evaluation of some nutritional and biochemical indicators in selecting salt-resistant tomato cultivars. Environ. Exp. Bot. **54**(3): 193-201.

Kchaou, H., Larbi, A., Gargouri, K., Chaieb, M., Morales, F., Msallem, M. 2010. Assessment of tolerance to NaCl salinity of five olive cultivars, based on growth characteristics and Na^+ and Cl^- exclusion mechanisms. Scientia Horticulturae **124**(3): 306-315.

Kerepesi, I. and Galiba, G. 2000. Osmotic and salt stress-induced alteration in soluble carbohydrate content in wheat seedlings. Crop Sci. **40**(2): 482-487.

Keser, M., Swenarton, J. T., Foertch, J. F. 2005. Effects of thermal input and climate change on growth of *Ascophyllum nodosum* (fucales, phaeophyceae) in eastern long island sound (USA). J. Sea Res. **54**(3): 211-220.

Khan, M. A., Ungar, I. A., Showalter, A. M. 1999. Effects of salinity on growth, ion content and osmotic relations in *Halopyrum mucronatum* (L.) stapf. J. Plant Nutr. **22**(1): 191-204.

Khan, M. A., Ungar, I. A., Showalter, A. M. 2000. Effects of salinity on growth, water relations and ion accumulation of the subtropical perennial halophyte, *Atriplex griffithii* var. stocksii. Annals of Botany **85**(2): 225-232.

Khan, W., Hiltz, D., Critchley, A. T., Prithiviraj, B. 2011. Bioassay to detect *Ascophyllum nodosum* extract-induced cytokinin-like activity in *Arabidopsis thaliana*. J. Appl. Phycol. **23**(3): 409-414.

Khan, W., Rayirath, U. P., Subramanian, S., Jithesh, M. N., Rayorath, P., Hodges, D. M., Critchley, A. T., Craigie, J. S., Norrie, J., Prithiviraj, B. 2009. Seaweed extracts as biostimulants of plant growth and development. J. Plant Growth Regul. **28**(4): 386-399.

Khatkar, D. and Kuhad, M. 2000. Short-term salinity induced changes in two wheat cultivars at different growth stages. Biol. Plant. **43**(4): 629-632.

Khavari-Nejad, R. and Mostofi, Y. 1998. Effects of NaCl on photosynthetic pigments, saccharides, and chloroplast ultrastructure in leaves of tomato cultivars. Photosynthetica **35**(1): 151-154.

Kim, T. H., Bohmer, M., Hu, H., Nishimura, N., Schroeder, J. I. 2010. Guard cell signal transduction network: Advances in understanding abscisic acid, CO_2, and Ca^{2+} signaling. Annu. Rev. Plant. Biol. **61**: 561-591.

Kochian, L. V. and Lucas, W. J. 2014. Plant mineral nutrient sensing and signaling. Journal of Integrative Plant Biology **56**(3): 190-191.

Kohlmeyer, J. and Kohlmeyer, E. 1972. Is *Ascophyllum nodosum* lichenized? Bot. Mar. **15**(2): 109-112.

Kronzucker, H. J. and Britto, D. T. 2011. Sodium transport in plants: A critical review. New Phytol. **189**(1): 54-81.

Kurban, H., Saneoka, H., Nehira, K., Adilla, R., Premachandra, G. S., Fujita, K. 1999. Effect of salinity on growth, photosynthesis and mineral composition in leguminous plant *Alhagi pseudoalhagi* (bieb.). Soil Sci. Plant Nutr. **45**(4): 851-862.

Li, Y. L. and Stanghellini, C. 2001. Analysis of the effect of EC and potential transpiration on vegetative growth of tomato. Scientia Horticulturae **89**(1): 9-21.

Lippert, K. and Galinski, E. A. 1992. Enzyme stabilization by ectoine-type compatible solutes: Protection against heating, freezing and drying. Appl. Microbiol. Biotechnol. **37**(1): 61-65.

Livak, K. J. and Schmittgen, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{\Delta\Delta CT}$ method. Methods **25**(4): 402-408.

Longstreth, D. J. and Nobel, P. S. 1979. Salinity effects on leaf anatomy consequences for photosynthesis. Plant Physiol. **63**(4): 700-703.

Longstreth, D. J., Bolaños, J. A., Smith, J. E. 1984. Salinity effects on photosynthesis and growth in *Alternanthera philoxeroides* (mart.) griseb. Plant Physiol. **75**(4): 1044-1047.

Lovelli, S., Scopa, A., Perniola, M., Di Tommaso, T., Sofo, A. 2012. Abscisic acid root and leaf concentration in relation to biomass partitioning in salinized tomato plants. J. Plant Physiol. **169**(3): 226-233.

Lu, C., Li, Y., Chen, A., Li, L., Zuo, J., Tian, H., Luo, Y., Zhu, B. 2010. LeERF1 improves tolerance to drought stress in tomato (*Lycopersicon esculentum*) and activates downstream stress-responsive genes. African Journal of Biotechnology **9**(38): 6294-6300.

Lutts, S., Majerus, V., Kinet, J. 1999. NaCl effects on proline metabolism in rice (*Oryza sativa*) seedlings. Physiol. Plantarum **105**(3): 450-458.

Ma, N., Zuo, Y., Liang, X., Yin, B., Wang, G., Meng, Q. 2013. The multiple stress-responsive transcription factor *SlNAC1* improves the chilling tolerance of tomato. Physiol. Plantarum **149**(4): 474-486.

McCree, K. 1986. Whole-plant carbon balance during osmotic adjustment to drought and salinity stress. Functional Plant Biology **13**(1): 33-43.

MacKinnon, S. L., Hiltz, D., Ugarte, R., Craft, C. A. 2010. Improved methods of analysis for betaines in *Ascophyllum nodosum* and its commercial seaweed extracts. J. Appl. Phycol. **22**(4): 489-494.

McCue, K. F. and Hanson, A. D. 1992. Salt-inducible betaine aldehyde dehydrogenase from sugar beet: CDNA cloning and expression. Plant Mol. Biol. **18**(1): 1-11.

Magdy, M., Mansour, F., Hasselt, P. R., Kuiper, P. J. 1994. Plasma membrane lipid alterations induced by NaCl in winter wheat roots. Physiol. Plantarum **92**(3): 473-478.

Mahajan, S. and Tuteja, N. 2005. Cold, salinity and drought stresses: An overview. Arch. Biochem. Biophys. **444**(2): 139-158.

Maiale, S., Sánchez, D. H., Guirado, A., Vidal, A., Ruiz, O. A. 2004. Spermine accumulation under salt stress. J. Plant Physiol. **161**(1): 35-42.

Mansour, M. and Stadelmann, E. 1994. NaCl-induced changes in protoplasmic characteristics of *Hordeum vulgare* cultivars differing in salt tolerance. Physiol. Plantarum **91**(3): 389-394.

Mansour, M. M. F. and Salama, K. H. 2004. Cellular basis of salinity tolerance in plants. Environ. Exp. Bot. **52**(2): 113-122.

Mäser, P., Eckelman, B., Vaidyanathan, R., Horie, T., Fairbairn, D. J., Kubo, M., Yamagami, M., Yamaguchi, K., Nishimura, M., Uozumi, N. 2002. Altered shoot/root Na^+ distribution and bifurcating salt sensitivity in *Arabidopsis* by genetic disruption of the Na^+ transporter *AtHKT1*. FEBS Lett. **531**(2): 157-161.

Mattioni, C., Lacerenza, N., Troccoli, A., Leonardis, A. d., Fonzo, N. d. 1997. Water and salt stress-induced alterations in proline metabolism of *Triticum durum* seedlings. Physiol. Plantarum **101**(4): 787-792.

Maurel, C., Verdoucq, L., Luu, D., Santoni, V. 2008. Plant aquaporins: Membrane channels with multiple integrated functions. Annu.Rev.Plant Biol. **59**: 595-624.

Metting, B., Zimmerman, W. J., Crouch, I., van Staden, J. 1990. Agronomic uses of seaweed and microalgae. Introduction of Applied Phycology.SPB, The Hague, 589-627.

Mishra, S. K., Subrahmanyam, D., Singhal, G. S. 1991. Interrelationship between salt and light stress on primary processes of photosynthesis. J. Plant Physiol. **138**(1): 92-96.

Mitsuya, S., Takeoka, Y., Miyake, H. 2000. Effects of sodium chloride on foliar ultrastructure of sweet potato (*Ipomoea batatas* lam.) plantlets grown under light and dark conditions *in vitro*. J. Plant Physiol. **157**(6): 661-667.

Mittler, R. 2002. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. **7**(9): 405-410.

Mittler, R., Herr, E. H., Orvar, B. L., Van Camp, W., Willekens, H., Inzé, D., Ellis, B. E. 1999. Transgenic tobacco plants with reduced capability to detoxify reactive oxygen intermediates are hyper-responsive to pathogen infection. Proceedings of the National Academy of Sciences **96**(24): 14165-14170.

Mittova, V., Guy, M., Tal, M., Volokita, M. 2004. Salinity up-regulates the antioxidative system in root mitochondria and peroxisomes of the wild salt-tolerant tomato species *Lycopersicon pennellii*. J. Exp. Bot. **55**(399): 1105-1113.

Mohammad, M., Shibli, R., Ajlouni, M., Nimri, L. 1998. Tomato root and shoot responses to salt stress under different levels of phosphorus nutrition. J. Plant Nutr. **21**(8): 1667-1680.

Mohanty, A., Kathuria, H., Ferjani, A., Sakamoto, A., Mohanty, P., Murata, N., Tyagi, A. 2002. Transgenics of an elite indica rice variety pusa basmati 1 harbouring the codA gene are highly tolerant to salt stress. Theor. Appl. Genet. **106**(1): 51-57.

Mok, D. W. and Mok, M. C. 2001. Cytokinin metabolism and action. Annual Review of Plant Biology **52**(1): 89-118.

Mulholland, B. J., Taylor, I. B., Jackson, A. C., Thompson, A. J. 2003. Can ABA mediate responses of salinity stressed tomato? Environ. Exp. Bot. **50**(1): 17-28.

Munns, R. 2002. Comparative physiology of salt and water stress. Plant, Cell Environ. **25**(2): 239-250.

Munns, R. 1993. Physiological processes limiting plant growth in saline soils: Some dogmas and hypotheses. Plant, Cell Environ. **16**(1): 15-24.

Munns, R. 2005. Genes and salt tolerance: Bringing them together. New Phytol. **167**(3): 645-663.

Munns, R. and Tester, M. 2008. Mechanisms of salinity tolerance. Annu.Rev.Plant Biol. **59**: 651-681.

Munns, R. and Termaat, A. 1986. Whole-plant responses to salinity. Functional Plant Biology **13**(1): 143-160.

Munns, R., James, R. A., Läuchli, A. 2006. Approaches to increasing the salt tolerance of wheat and other cereals. J. Exp. Bot. **57**(5): 1025-1043.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plantarum **15**(3): 473-497.

Nabati, D., Schmidt, R., Parrish, D. 1994. Alleviation of salinity stress in Kentucky bluegrass by plant growth regulators and iron. Crop Sci. **34**(1): 198-202.

Nabati, D. A. 1991. Responses of Two Grass Species to Plant Growth Regulators, Fertilizer N, Chelated Fe, Salinity and Water Stress. Ph.D thesis.

Nair, P., Kandasamy, S., Zhang, J., Ji, X., Kirby, C., Benkel, B., Hodges, M. D., Critchley, A. T., Hiltz, D., Prithiviraj, B. 2012. Transcriptional and metabolomic analysis of *Ascophyllum nodosum* mediated freezing tolerance in *Arabidopsis thaliana*. BMC Genomics **13**(1): 1-23.

Nanawati, G. and Maliwal, G. 1974. Note on the effect of salts on the growth, mineral nutrition and quality of tomato (*Lycopersicon esculentum* mill.). Indian J. Agric. Sci. **43**: 612-614.

Niu, X., Bressan, R. A., Hasegawa, P. M., Pardo, J. M. 1995. Ion homeostasis in NaCl stress environments. Plant Physiol. **109**(3): 735.

Noaman, M. M., Dvorak, J., Dong, J. 2002. Genes inducing salt tolerance in wheat, lophopyrum elongatum and amphiploid and their responses to ABA under salt stress. Pages 139-144 *in:* Prospects for saline agriculture. Springer.

Olias, R., Eljakaoui, Z., Li, J., de Morales, P. A., Marín-Manzano, M. C., Pardo, J. M., Belver, A. 2009. The plasma membrane Na /H antiporter SOS1 is essential for salt tolerance in tomato and affects the partitioning of Na between plant organs. Plant, Cell Environ. **32**(7): 904-916.

Orthen, B., Popp, M., Smirnoff, N. 1994. Hydroxyl radical scavenging properties of cyclitols. Proceedings-Royal Society of Edinburgh b **102**: 269-269.

Osmond, C., Austin, M., Berry, J., Billings, W., Boyer, J., Dacey, J., Nobel, P., Smith, S., Winner, W. 1987. Stress physiology and the distribution of plants. Bioscience **37**(1): 38-48.

Ouyang, B., Yang, T., Li, H., Zhang, L., Zhang, Y., Zhang, J., Fei, Z., Ye, Z. 2007. Identification of early salt stress response genes in tomato root by suppression subtractive hybridization and microarray analysis. J. Exp. Bot. **58**(3): 507-520.

Papadopoulos, I. and Rendig, V. 1983. Interactive effects of salinity and nitrogen on growth and yield of tomato plants. Plant Soil **73**(1): 47-57.

Parida, A., Das, A. B., Das, P. 2002. NaCl stress causes changes in photosynthetic pigments, proteins, and other metabolic components in the leaves of a true mangrove, *Bruguiera parviflora*, in hydroponic cultures. Journal of Plant Biology **45**(1): 28-36.

Parida, A. K. and Das, A. B. 2005. Salt tolerance and salinity effects on plants: A review. Ecotoxicol. Environ. Saf. **60**(3): 324-349.

Parida, A. K., Das, A., Mittra, B. 2004. Effects of salt on growth, ion accumulation, photosynthesis and leaf anatomy of the mangrove, *Bruguiera parviflora*. Trees **18**(2): 167-174.

Pardo, J. M. and Rubio, F. 2010. Naþ and Kþ transporters in plant signaling. Transporters and Pumps in Plant Signaling **7**: 65.

Pasternak, D. 1987. Salt tolerance and crop production-a comprehensive approach. Annu. Rev. Phytopathol. **25**(1): 271-291.

Paul, M. J. and Foyer, C. H. 2001. Sink regulation of photosynthesis. J. Exp. Bot. **52**(360): 1383-1400.

Pedranzani, H., Racagni, G., Alemano, S., Miersch, O., Ramírez, I., Peña-Cortés, H., Taleisnik, E., Machado-Domenech, E., Abdala, G. 2003. Salt tolerant tomato plants show increased levels of jasmonic acid. Plant Growth Regulation **41**(2): 149-158.

Pessarakli M, Szabolcs I, 1999. Soil salinity and sodicity as particular plant/crop stress factors. In: Pessarakli M (Ed) Handbook of plant and crop stress. Dekker, New York, pp 1–16.

Polle, A. 2001. Dissecting the superoxide dismutase-ascorbate-glutathione-pathway in chloroplasts by metabolic modeling. Computer simulations as a step towards flux analysis. Plant Physiol. **126**(1): 445-462.

Popova, L. P., Stoinova, Z. G., Maslenkova, L. T. 1995. Involvement of abscisic acid in photosynthetic process in *Hordeum vulgare* L. during salinity stress. J. Plant Growth Regul. **14**(4): 211-218.

Popp, M., Larher, F., Weigel, P. 1985. Osmotic adaption in Australian mangroves. Pages 247-253 *in:* Ecology of coastal vegetation. Springer,

Prasad, S., Bagali, P., Hittalmani, S., Shashidhar, H. 2000. Molecular mapping of quantitative trait loci associated with seedling tolerance to salt stress in rice (oryza sativa L.). Curr. Sci. **78**(2): 162-164.

Qadir, M., Oster, J., Schubert, S., Noble, A., Sahrawat, K. 2007. Phytoremediation of sodic and Saline‐Sodic soils. Adv. Agron. **96**: 197-247.

Qiu, Y. and Yu, D. 2009. Over-expression of the stress-induced *OsWRKY45* enhances disease resistance and drought tolerance in *Arabidopsis*. Environ. Exp. Bot. **65**(1): 35-47.

Rahman, M. and Punja, Z. K. 2005. Biochemistry of ginseng root tissues affected by rusty root symptoms. Plant Physiology and Biochemistry **43**(12): 1103-1114.

Rai, A., Tripathi, P., Dwivedi, S., Dubey, S., Shri, M., Kumar, S., Tripathi, P. K., Dave, R., Kumar, A., Singh, R. 2011. Arsenic tolerances in rice (*Oryza sativa*) have a predominant role in transcriptional regulation of a set of genes including sulphur assimilation pathway and antioxidant system. Chemosphere **82**(7): 986-995.

Rajesh, A., Arumugam, R., Venkatesalu, V. 1998. Growth and photosynthetic characteristics of *Ceriops roxburghiana* under NaCl stress. Photosynthetica **35**(2): 285-287.

Rayorath, P., Jithesh, M. N., Farid, A., Khan, W., Palanisamy, R., Hankins, S. D., Critchley, A. T., Prithiviraj, B. 2008. Rapid bioassays to evaluate the plant growth promoting activity of *Ascophyllum nodosum* (L.) le jol. using a model plant, *Arabidopsis thaliana* (L.) heynh. J. Appl. Phycol. **20**(4): 423-429.

Reddy, M., Sanish, S., Iyengar, E. 1992. Photosynthetic studies and compartmentation of ions in different tissues of *Salicornia brachiata* under saline conditions. Photosynthetica **26**.

Reddy, M., Sanish, S., Iyengar, E. 1993. Compartmentation of ions and organic compounds in *Salicornia brachiata* roxb. Biol. Plant. **35**(4): 547-553.

Rengasamy, P. 2002. Transient salinity and subsoil constraints to dryland farming in Australian sodic soils: An overview. Animal Production Science **42**(3): 351-361.

Rhodes, D. and Hanson, A. 1993. Quaternary ammonium and tertiary sulfonium compounds in higher plants. Annual Review of Plant Biology **44**(1): 357-384.

Rivero, R. M., Mestre, T. C., Mittler, R., Rubio, F., Garcia-Sanchez, F., Martinez, V. 2013. The combined effect of salinity and heat reveals a specific physiological, biochemical and molecular response in tomato plants. Plant, Cell Environ.

Romero-Aranda, R., Soria, T., Cuartero, J. 2001. Tomato plant-water uptake and plant-water relationships under saline growth conditions. Plant Science **160**(2): 265-272.

Saneoka, H., Shiota, K., Kurban, H., Chaudhary, M. I., Premachandra, G. S., Fujita, K. 1999. Effect of salinity on growth and solute accumulation in two wheat lines differing in salt tolerance. Soil Sci. Plant Nutr. **45**(4): 873-880.

Sawahel, W. A. and Hassan, A. H. 2002. Generation of transgenic wheat plants producing high levels of the osmoprotectant proline. Biotechnol. Lett. **24**(9): 721-725.

Sarkar, D., Bhowmik, P. C., Kwon, Y., Shetty, K. 2009. Clonal response to cold tolerance in creeping bentgrass and role of proline-associated pentose phosphate pathway. Bioresour. Technol. **100**(21): 5332-5339.

Scandalios, J. G., Guan, L., Polidoros, A. N. 1997. Catalases in plants: Gene structure, properties, regulation, and expression. Cold Spring Harbor Monogr. Ser. **34**: 343-406.

Schofield, R., Thomas, D., Kirkby, M. 2001. Causal processes of soil salinization in Tunisia, Spain and Hungary. Land Degrad. Dev. **12**(2): 163-181.

Schofield, R. and Kirkby, M. 2003. Application of salinization indicators and initial development of potential global soil salinization scenario under climatic change. Global Biogeochem. Cycles **17**(3).

Schroeder, J. I., Kwak, J. M., Allen, G. J. 2001. Guard cell abscisic acid signalling and engineering drought hardiness in plants. Nature **410**(6826): 327-330.

Schroeder, J. I., Allen, G. J., Hugouvieux, V., Kwak, J. M., Waner, D. 2001. Guard cell signal transduction. Annual Review of Plant Biology **52**(1): 627-658.

Seemann, J. R. and Critchley, C. 1985. Effects of salt stress on the growth, ion content, stomatal behaviour and photosynthetic capacity of a salt-sensitive species, phaseolus vulgaris L. Planta **164**(2): 151-162.

Singh, S., Sharma, H., Goswami, A., Datta, S., Singh, S. 2000. In vitro growth and leaf composition of grapevine cultivars as affected by sodium chloride. Biol. Plant. **43**(2): 283-286.

Shabala, S. and Cuin, T. A. 2008. Potassium transport and plant salt tolerance. Physiol. Plantarum **133**(4): 651-669.

Shahbaz, M., Ashraf, M., Al-Qurainy, F., Harris, P. 2012. Salt tolerance in selected vegetable crops. Crit. Rev. Plant Sci. **31**(4): 303-320.

Shannon, M. C. 1997. Adaptation of plants to salinity. Adv. Agron. **60**: 75-120.

Shannon, M. and Grieve, C. 1998. Tolerance of vegetable crops to salinity. Scientia Horticulturae **78**(1): 5-38.

Shinozaki, K. and Yamaguchi-Shinozaki, K. 1997. Gene expression and signal transduction in water-stress response. Plant Physiol. **115**(2): 327.

Smirnoff, N. 2000. Ascorbic acid: Metabolism and functions of a multi-faceted molecule. Curr. Opin. Plant Biol. **3**(3): 229-235.

Smith, A. M. and Stitt, M. 2007. Coordination of carbon supply and plant growth. Plant, Cell Environ. **30**(9): 1126-1149.

Soussi, M., Ocana, A., Lluch, C. 1998. Effects of salt stress on growth, photosynthesis and nitrogen fixation in chick-pea (*Cicer arietinum* L.). J. Exp. Bot. **49**(325): 1329-1337.

Staal, M., Maathuis, F. J., Elzenga, J. T. M., Overbeek, J. H. M., Prins, H. 1991. Na /H antiport activity in tonoplast vesicles from roots of the salt ‐ tolerant plantago maritima and the salt ‐ sensitive plantago media. Physiol. Plantarum **82**(2): 179-184.

Stewart, G. and Lee, J. 1974. The role of proline accumulation in halophytes. Planta **120**(3): 279-289.

Stirk, W., Novák, O., Strnad, M., Van Staden, J. 2003. Cytokinins in macroalgae. Plant Growth Regulation **41**(1): 13-24.

Subramanian, S. 2008. Studies on *Ascophyllum nodosum* extract elicited disease resistance in *Arabidopsis thaliana* against *Pseudomonas syringae* pv. tomato DC3000. Dalhousie University (Canada), Master Thesis.

Subramanian, S., Sangha, J. S., Gray, B. A., Singh, R. P., Hiltz, D., Critchley, A. T., Prithiviraj, B. 2011. Extracts of the marine brown macroalga, *Ascophyllum nodosum*, induce jasmonic acid dependent systemic resistance in *Arabidopsis thaliana* against *Pseudomonas syringae* pv. tomato DC3000 and *Sclerotinia sclerotiorum*. Eur. J. Plant Pathol. **131**(2): 237-248.

Suhayda, C. G., Giannini, J. L., Briskin, D. P., Shannon, M. C. 1990. Electrostatic changes in *Lycopersicon esculentum* root plasma membrane resulting from salt stress. Plant Physiol. **93**(2): 471-478.

Taiz, L. and Zeiger, E. 2002. Plant physiology. EE.UU.California: Sinauer, 3rd Ed: 41-99.

Tanji, K.K. and Kielen, N.C. 2002. Agricultural drainage water management in arid and semi-arid areas: FAO Irrigation and Drainage Paper 6. Food and Agriculture Organization of the United Nations, Rome.

Tanji, K. K. 1990. Agricultural salinity assessment and management. Amer Society of Civil Engineers, **71**.

Tardieu, F., Granier, C., Muller, B. 2011. Water deficit and growth. co-ordinating processes without an orchestrator? Curr. Opin. Plant Biol. **14**(3): 283-289.

Tavakkoli, E., Rengasamy, P., McDonald, G. K. 2010. High concentrations of Na^+ and Cl^- ions in soil solution have simultaneous detrimental effects on growth of faba bean under salinity stress. J. Exp. Bot. **61**(15): 4449-4459.

Tavakkoli, E., Fatehi, F., Coventry, S., Rengasamy, P., McDonald, G. K. 2011. Additive effects of Na^+ and Cl^- ions on barley growth under salinity stress. J. Exp. Bot. **62**(6): 2189-2203.

Temple, W. and Bomke, A. 1989. Effects of kelp (*Macrocystis integrifolia* and *Ecklonia maxima*) foliar applications on bean crop growth. Plant Soil **117**(1): 85-92.

Tester, M. and Davenport, R. 2003. Na^+ tolerance and na+ transport in higher plants. **Ann. Bot. 91(5): 503-527.**

Thomas, J. C., McElwain, E. F., Bohnert, H. J. 1992. Convergent induction of osmotic stress-responses abscisic acid, cytokinin, and the effects of NaCl. Plant Physiol. **100**(1): 416-423.

Tomato Genome Consortium. 2012. The tomato genome sequence provides insights into fleshy fruit evolution. Nature **485**(7400): 635-641.

Torrecillas, A., Guillaume, C., Alarcón, J. J., Ruiz-Sánchez, M. C. 1995. Water relations of two tomato species under water stress and recovery. Plant Science **105**(2): 169-176.

Tunçtürk, M., Tunçtürk, R., Yildirim, B., Çiftçi, V. 2011. Changes of micronutrients, dry weight and plant development in canola (*Brassica napus* L.) cultivars under salt stress. African J Biotechnol **10**: 3726-3730.

Tuteja, N., Ahmad, P., Panda, B. B., Tuteja, R. 2009. Genotoxic stress in plants: Shedding light on DNA damage, repair and DNA repair helicases. Mutation Research/Reviews in Mutation Research **681**(2): 134-149.

Ugarte, R. A. and Sharp, G. 2001. 6. A new approach to seaweed management in eastern Canada: The case of *Ascophyllum nodosum*. Cah. Biol. Mar. **42**(1/2): 63-70.

USDA-ARS. 2008. Research Databases. Bibliography on Salt Tolerance. George E. Brown, Jr. Salinity Lab. US Dep. Agric., Agric. Res. Serv. Riverside, CA. http://www.ars.usda.gov/Services/docs.htm?docid=8908

Vaidyanathan, R., Kuruvilla, S., Thomas, G. 1999. Characterization and expression pattern of an abscisic acid and osmotic stress responsive gene from rice. Plant Science **140**(1): 21-30.

Villalta, I., Reina-Sánchez, A., Bolarín, M., Cuartero, J., Belver, A., Venema, K., Carbonell, E., Asins, M. 2008. Genetic analysis of Na and K concentrations in leaf and stem as physiological components of salt tolerance in tomato. Theor. Appl. Genet. **116**(6): 869-880.

Vogel, C. and Marcotte, E. M. 2012. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. Nature Reviews Genetics **13**(4): 227-232.

Wally, O. S., Critchley, A. T., Hiltz, D., Craigie, J. S., Han, X., Zaharia, L. I., Abrams, S. R., Prithiviraj, B. 2013. Regulation of phytohormone biosynthesis and accumulation in *Arabidopsis* following treatment with commercial extract from the marine macroalga *Ascophyllum nodosum*. J. Plant Growth Regul. **32**(2): 324-339.

Wang, D., Wang, H., Han, B., Wang, B., Guo, A., Zheng, D., Liu, C., Chang, L., Peng, M., Wang, X. 2012. Sodium instead of potassium and chloride is an important macronutrient to improve leaf succulence and shoot development for halophyte *Sesuvium portulacastrum*. Plant Physiology and Biochemistry **51**: 53-62.

Wang, Y. and Nii, N. 2000. Changes in chlorophyll, ribulose bisphosphate carboxylase-oxygenase, glycine betaine content, photosynthesis and transpiration in amaranthus tricolor leaves during salt stress. Journal of Horticultural Science and Biotechnology **75**(6): 623-627.

Weeraddana, C. D. S. 2012. Extracts of the Brown Seaweed, *Ascophyllum nodosum*, Effect *Arabidopsis thaliana–Myzus persicae* Interaction. Master Thesis.

Wei, J., Tirajoh, A., Effendy, J., Plant, A. L. 2000. Characterization of salt-induced changes in gene expression in tomato (*Lycopersicon esculentum*) roots and the role played by abscisic acid. Plant Science **159**(1): 135-148.

White, P. J. and Broadley, M. R. 2001. Chloride in soils and its uptake and movement within the plant: A review. Annals of Botany **88**(6): 967-988.

Wilkinson, S. and Davies, W. J. 2002. ABA - based chemical signaling: The co - ordination of responses to stress in plants. Plant, Cell Environ. **25**(2): 195-210.

Wu, S., Ding, L., Zhu, J. 1996. SOS1, a genetic locus essential for salt tolerance and potassium acquisition. The Plant Cell Online **8**(4): 617-627.

Xiong, L., Ishitani, M., Lee, H., Zhu, J. 2001. The *Arabidopsis* LOS5/ABA3 locus encodes a molybdenum cofactor sulfurase and modulates cold stress– and osmotic stress–responsive gene expression. The Plant Cell Online **13**(9): 2063-2083.

Xu, H., Deckert, R. J., Garbary, D. J. 2008. *Ascophyllum* and its symbionts. X. ultrastructure of the interaction between *A. nodosum* (phaeophyceae) and *Mycophycias ascophylli* (ascomycetes). Botany **86**(2): 185-193.

Yamaguchi, T. and Blumwald, E. 2005. Developing salt-tolerant crop plants: Challenges and opportunities. Trends Plant Sci. **10**(12): 615-620.

Yan, J. 1993. Influence of Plant Growth Regulators on Turfgrass Polar Lipid Composition, Tolerance to Drought and Salinity Stresses, and Nutrient Efficiency. PhD Thesis.

Yang, T. and Poovaiah, B. 2002. Hydrogen peroxide homeostasis: Activation of plant catalase by calcium/calmodulin. Proceedings of the National Academy of Sciences **99**(6): 4097-4102.

Yeo, A., Flowers, S., Rao, G., Welfare, K., Senanayake, N., Flowers, T. 1999. Silicon reduces sodium uptake in rice (*Oryza sativa* L.) in saline conditions and this is accounted for by a reduction in the transpirational bypass flow. Plant, Cell Environ. **22**(5): 559-565.

Zapata, P. J., Botella, M. Á., Pretel, M. T., Serrano, M. 2007. Responses of ethylene biosynthesis to saline stress in seedlings of eight plant species. Plant Growth Regulation **53**(2): 97-106.

Zhang, H. and Blumwald, E. 2001. Transgenic salt-tolerant tomato plants accumulate salt in foliage but not in fruit. Nat. Biotechnol. **19**(8): 765-768.

Zhang, J. Z., Creelman, R. A., Zhu, J. 2004. From laboratory to field. Using information from *Arabidopsis* to engineer salt, cold, and drought tolerance in crops. Plant Physiol. **135**(2): 615-621.

Zhang, L., Tian, L. H., Zhao, J. F., Song, Y., Zhang, C. J., Guo, Y. 2009. Identification of an apoplastic protein involved in the initial phase of salt stress response in rice root by two-dimensional electrophoresis. Plant Physiol. **149**(2): 916-928.

Zhang, X. 1997. Influence of Plant Growth Regulators on Turfgrass Growth, Antioxidant Status, and Drought Tolerance. Ph.D Thesis.

Zhang, X. and Schmidt, R. 1999. Antioxidant response to hormone-containing product in Kentucky bluegrass subjected to drought. Crop Sci. **39**(2): 545-551.

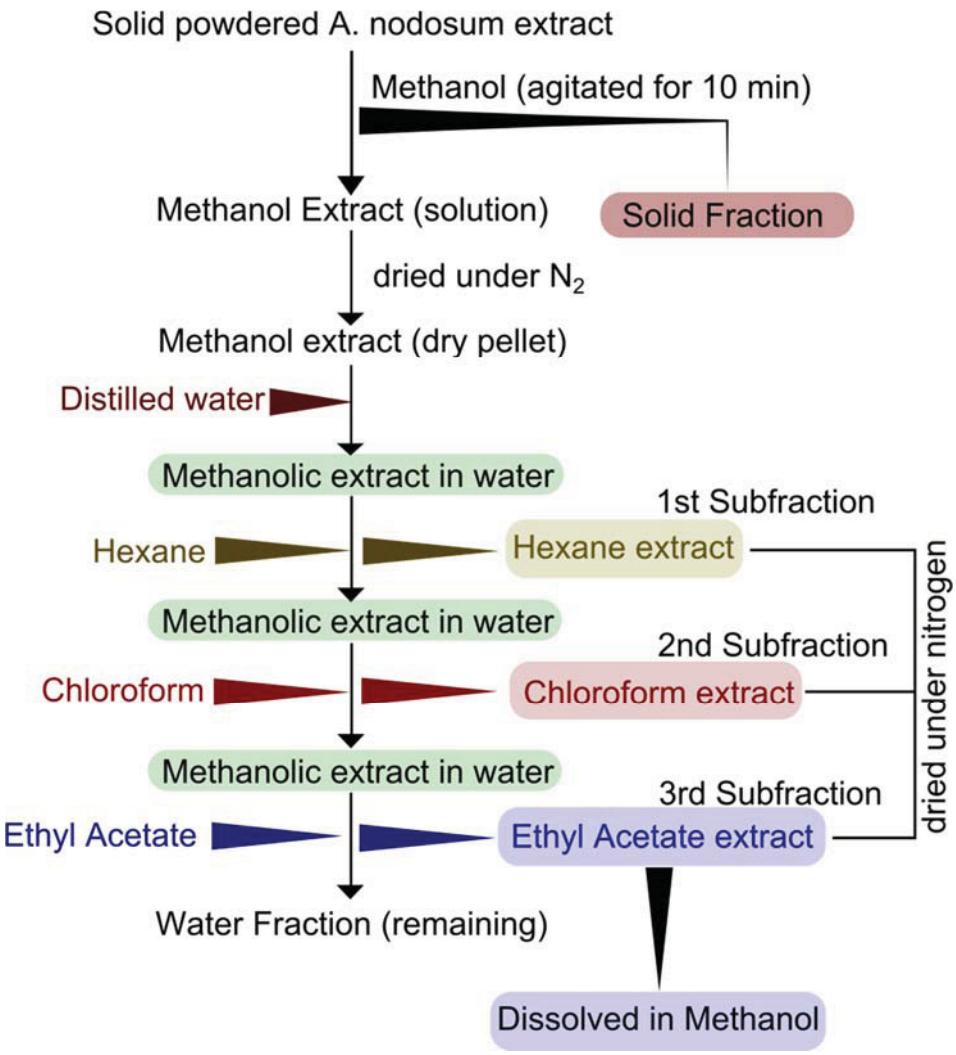
Zhu, J. 2003. Regulation of ion homeostasis under salt stress. Curr. Opin. Plant Biol. **6**(5): 441-445.

Zhou, J., Wang, X., Jiao, Y., Qin, Y., Liu, X., He, K., Chen, C., Ma, L., Wang, J., Xiong, L. 2007. Global genome expression analysis of rice in response to drought and high-salinity stresses in shoot, flag leaf, and panicle. Plant Mol. Biol. **63**(5): 591-608.

Zhu, J. 2002. Salt and drought stress signal transduction in plants. Annual Review of Plant Biology **53**: 247.

Zhu, J., Hasegawa, P. M., Bressan, R. A., Bohnert, H. J. 1997. Molecular aspects of osmotic stress in plants. Crit. Rev. Plant Sci. **16**(3): 253-277.

APPENDIX I: ORGANIC SUB-FRACTIONATION OF ANE



APPENDIX II: ANALYSIS OF VARIANCE

Effect of Ethyl acetate organic fraction of *Ascophyllum nodosum* extract on Leaf area, root length and root area of tomato

ANOVA Leaf Area	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatments	3	412	137	100.8	4.11e-15
Residuals	28	38.2	1.36		

Treatments	Compact letter display
100mM NaCl	a
Control	c
EtOAc- ANE	c
EtOAc-ANE-100mM NaCl	b

ANOVA Root length	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatments	3	5381	1793.5	24.48	5.58e-08
Residuals	28	2052	73.3		

Treatments	Compact letter display
100mM NaCl	a
Control	b
EtOAc- ANE	b
EtOAc-ANE-100mM NaCl	b

ANOVA Root area	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatments	3	37.3	12.434	5.726	0.00347
Residuals	28	60.8	2.172		

Treatments	Compact letter display
100mM NaCl	a
Control	ab
EtOAc- ANE	b
EtOAc-ANE-100mM NaCl	a

Effect of commercial *Ascophyllum nodosum* extract on Leaf area, root length, root area and fresh weight of tomato plants

Mean squares

Name	n	Leaf area (cm^2)	Root length (cm)	Root surface area (cm^2)	Fresh Weight, (g)
0 g/L ANE	18	127.5	894.5	125	17.56
1 g/L ANE	18	127.8	886.1	126.2	17.54
0.3 g/L ANE	18	127.8	856.8	125.8	17.6
	.				
Grand Mean	.	127.7	879.1	125.7	17.56
SEM	.	1.57	20.53	1.522	0.2502
F pr	.				
ANE	-p	ns	ns	ns	ns
.. linANE	-p	ns	ns	ns	ns
.. quadANE	-p	ns	ns	ns	ns

Name	n	Leaf area (cm^2)	Root length (cm)	Root surface area (cm^2)	Fresh Weight, (g)
0mM Salt	18	146.1	1011	146.3	19.8
100mM Salt	18	127.5	857.5	126.3	17.73
200mM Salt	18	109.5	768.5	104.4	15.16
	.				
Grand Mean	.	127.7	879.1	125.7	17.56
SEM	.	1.57	20.53	1.522	0.2502
F pr	.				
Salt	-p	<0.001	<0.001	<0.001	<0.001
.. linS	-p	<0.001	<0.001	<0.001	<0.001
.. quadS	-p	ns	ns	ns	ns

Name	n	Leaf area (cm^2)	Root length (cm)	Root surface area (cm2)	Fresh Weight, (g)
0 g/L ANE 0mM Salt	6	144.3	1062	145.3	19.32
0 g/L ANE 100mM Salt	6	127.3	859.6	127.7	17.68
0 g/L ANE 200mM Salt	6	110.9	762.1	101.9	15.68
1 g/L ANE 0mM Salt	6	143.8	1024	147.5	19.93
1 g/L ANE 100mM Salt	6	125.5	855.5	125.4	17.92
1 g/L ANE 200mM Salt	6	114.1	778.4	105.9	14.76
0.3 g/L ANE 0mM Salt	6	150.2	948.1	146.1	20.14
0.3 g/L ANE 100mM Salt	6	129.7	857.5	125.7	17.61
0.3 g/L ANE 200mM Salt	6	103.5	764.9	105.5	15.05
	.				
Grand Mean	.	127.7	879.1	125.7	17.56
SEM	.	2.719	35.56	2.635	0.4334
F pr	.				
ANE.Salt	-p	0.025	ns	ns	ns
.. linANE.linS	-p	ns	ns	ns	ns
.. quadANE.linS	-p	0.005	ns	ns	ns
.. linANE.quadS	-p	ns	ns	ns	ns
.. quadANE.quadS	-p	ns	ns	ns	ns

Source	d.f.	Leaf area (cm2)	Root length (cm)	Root surface area (cm2)	Fresh Weight, (g)
Block stratum	5	75.95	9398	159.3	1.369
Col stratum	2	68.12	32857	190.8	3.531
Block.Col.Row.fU stratum	*	*	*	*	*
ANE	2	0.4439	7035	7.431	0.01852
.. linANE	1	0.4328	2.761	12.75	0.01146
.. quadANE	1	0.455	14068	2.113	0.02558
Salt	2	6025	271850	7884	97.01
.. linS	1	12049	531060	15757	193.2
.. quadS	1	1.08	12639	9.931	0.7846
ANE.Salt	4	139.7	6789	19.43	1.27
.. linANE.linS	1	85.82	1250	2.172	2.569
.. quadANE.linS	1	392.5	19125	9.464	1.825
.. linANE.quadS	1	36.41	91.59	48.57	0.4784
.. quadANE.quadS	1	44.25	6688	17.51	0.2068
Residual	37	44.37	7585	41.67	1.127
Total	52	*	*	*	*

ANOVA

Source	d.f.	Leaf area (cm^2)	Root length (cm)	Root surface area (cm^2)	Fresh Weight, (g)
Block stratum	5	.	.	.	.
Col stratum	2	.	.	.	.
Block.Col.Row.fU stratum	*	.	.	.	.
ANE	2	ns	ns	ns	ns
.. linANE	1	ns	ns	ns	ns
.. quadANE	1	ns	ns	ns	ns
Salt	2	<0.001	<0.001	<0.001	<0.001
.. linS	1	<0.001	<0.001	<0.001	<0.001
.. quadS	1	ns	ns	ns	ns
ANE.Salt	4	0.025	ns	ns	ns
.. linANE.linS	1	ns	ns	ns	ns
.. quadANE.linS	1	0.005	ns	ns	ns
.. linANE.quadS	1	ns	ns	ns	ns
.. quadANE.quadS	1	ns	ns	ns	ns
Residual	37	.	.	.	.
Total	52	.	.	.	.

APPENDIX III: MINERAL COMPOSITION OF LONG ASHTON SOLUTION (LANS) AND INORGANIC CONTROL

Compound	LANS 0.1x Final conc'n (ppm=mg/L)	Modified LANS (inorganic control) 0.1x Final conc'n (ppm=mg/L)
KNO_3	K = 15.6 N = 5.6	K = 15.6 N = 5.6
$Ca(NO_3)_2\ 4H_2O$	Ca = 16.6 N = 11.2	Ca = 3.3 N = 2.2
$MgSO_4\ 7H_2O$	Mg = 3.6 S = 4.8	Mg = 3.6 S = 4.8
$NaH_2PO_4\ H_2O$	P = 4.1 Na = 3.1	P = 4.1 Na = 3.1
Fe-citrate H_2O	Fe = 0.28	Fe = 0.28
$MnSO_4\ H_2O$	Mn = 0.05	Mn = 0.05
$CuSO_4\ 5H_2O$	Cu = 0.003	Cu = 0.003
$ZnSO_4\ 7H_2O$	Zn = 0.013	Zn = 0.013
H_3BO_3	B = 0.032	B = 0.032
$Na_2MoO_4\ 2H_2O$	Mo = 0.005	Mo = 0.005
NaCl	Cl = 0.36 Na = 0.23	Cl = 0.36 Na = 0.23
$CoSO_4\ 7H_2O$	Co = 0.0012	Co = 0.0012
KCl	-	K = 65 Cl = 60
KOH	-	K = 65

Source: Acadian Seaplants Limited, Canada